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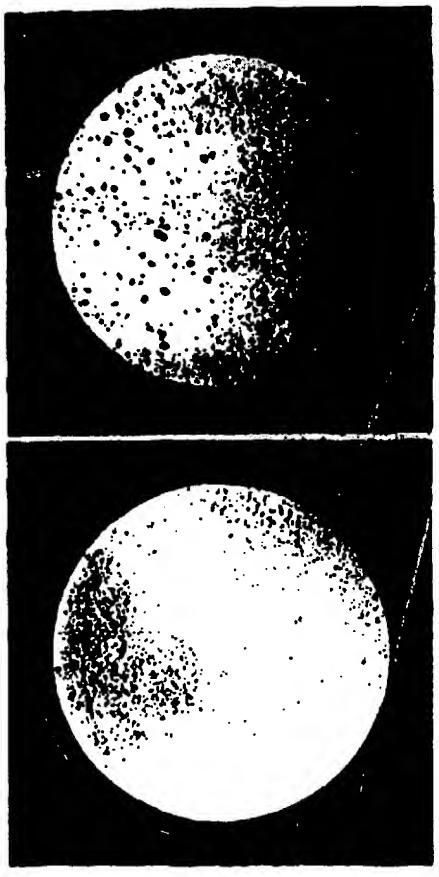
(54) Title: NEW PHARMACEUTICALS FOR MODULATING HORMONE RESPONSIVENESS

(57) Abstract

This invention relates to isolated and purified proteins, such as calreticulin and mimetics and inhibitors of calreticulin, for a novel use of modulating hormone responsiveness. These proteins are useful in gene therapy and in manufacturing pharmaceuticals for treating a variety of diseases, including cancer, osteoporosis and chronic inflammatory disease. The proteins include or bind to an amino acid sequence KXFFYR, wherein X is either G, A or V and Y is either K or R. This sequence is present in the DNA-binding domain, and is critical for the DNA binding activity, of a variety of hormone receptors, including glucocorticoid receptor, minerolcorticoid receptor, androgen receptor, progesterone receptor, estrogen receptor, retinoic acid receptor, thyroid hormone receptor and vitamin D receptor. Proteins which bind to this sequence may inhibit hormone receptor induced gene transcription. Proteins which include this sequence may promote hormone receptor induced gene transcription. The invention includes isolated DNA molecules for these proteins, methods of treating diseases using these proteins, synthetic peptides and their mimetics, and kits containing these proteins, synthetic peptides or their mimetics.



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NEW PHARMACEUTICALS FOR MODULATING HORMONE RESPONSIVENESS

BACKGROUND OF THE INVENTION

This invention relates to isolated and purified proteins, such as calreticulin and mimetics of calreticulin, for a novel use of modulating hormone responsiveness. These proteins are useful in gene therapy and in manufacturing pharmaceuticals for treating a variety of diseases, including cancer, osteoporosis and chronic inflammatory disease. The proteins include or bind to an amino acid sequence KXFFYR, wherein X is either G, A or V and Y is either K or R. This sequence is present in the DNA-binding domain, and is critical for the DNA binding activity, of a variety of hormone receptors, including glucocorticoid receptor, mineralcorticoid receptor, androgen receptor, progesterone receptor, estrogen receptor, retinoic acid receptor, thyroid hormone receptor and vitamin D receptor. Proteins which bind to this sequence inhibit hormone receptor induced gene transcription. Proteins which include this sequence promote hormone receptor induced gene transcription. The invention includes isolated DNA molecules for these proteins, methods of treating diseases using these proteins, synthetic peptides and their mimetics, and kits containing these proteins, synthetic peptides or their mimetics.

The physiology of many organs in mammals is regulated by hormones. These hormones include steroid hormones, thyroid hormones, metabolites of vitamins, such as all trans retinoic acid, 9-cis retinoic acid, vitamin D and its metabolite 1,25 dihydroxyvitamin D3. These hormones are proteins and bind to intracellular receptors which regulate expression of genes (O'Malley, 1990).

There are a variety of receptors which respond to hormones. Osteoblasts and osteoclasts respond to steroid hormones, vitamin D and retinoic acid. Mammary epithelial cells and breast carcinoma cells respond to estrogens, progesterone, retinoic acid and glucocorticoids. Lymphocytes respond to glucocorticoids.

The response of receptors to hormones is particularly important in the development of a number of diseases, including cancer, osteoporosis and chronic inflammatory disease. For example, the vitamin D receptor is strongly implicated in the evolution of osteoporosis (Morrison *et al.*, 1994).

The hormone receptor family is called the nuclear hormone receptor family and consists not only of receptors whose ligands are known, but also of an increasing number of orphan receptors whose ligands are unknown (O'Malley, 1990).

The nuclear hormone receptors can be divided into several domains which include the hormone (ligand) binding domain, the DNA-binding domain and the transactivation domain (O'Malley, 1990). The DNA-binding domain consists of two zinc fingers and is responsible for the receptor's binding to the DNA response elements which are found in the promoter and enhancer regions of the genes whose expression are regulated by these receptors. Once a hormone binds to its receptor, the receptor binds to the DNA thereby inducing gene transcription.

Proteins which modulate hormone receptor induced gene transcription are poorly understood. Such proteins are present in the nucleus of the cell and inhibit or promote the binding of a hormone to its receptor.

To help design pharmaceuticals and therapies for certain diseases, one must understand the function of certain intracellular proteins and their role in modulating hormone responsiveness. Isolation and purification of these proteins would help in assessing whether they inhibit or promote hormone receptor induced gene transcription. Once such proteins are isolated, manipulation of such proteins could further inhibit or promote hormone receptor induced gene transcription. Synthetic peptides which bind to such proteins could be used to promote hormone receptor induced gene transcription. Pharmaceuticals including such peptides or their mimetics could be used to inhibit hormone receptor induced gene transcription. Gene therapy could be used to inhibit or promote hormone receptor induced gene transcription.

A need exists to identify amino acid sequences that are conserved in hormone receptors, so that particular peptides and proteins may be designed and used in modulating hormone responsiveness. This would lead to improved methods of treating a variety of diseases, disorders and abnormal physical states in a mammals by regulating hormone receptor induced gene transcription in mammalian cells.

One protein that may be used in modulating hormone responsiveness is calreticulin. Calreticulin was initially identified as the major Ca^{2+}-storage protein in the sarcoplasmic reticulum of skeletal muscle (Ostwald and MacLennan, 1974). Subsequent work has revealed that the protein can also be detected in the endoplasmic reticulum of non-muscle tissues (Fliegel et al., 1989; Opas et al., 1991). Calreticulin has been considered to be a resident protein of the endoplasmic reticulum of a cell, where it is thought to behave as a calcium binding protein due to its high capacity calcium binding properties (Michalak *et al.*, 1992). Calreticulin possesses many diverse functional domains such as high affinity, low capacity- and low affinity, high capacity-Ca^{2+}-binding sites, a C-terminal KDEL endoplasmic reticulum retention signal, and a nuclear localization signal (Michalak et al., 1992).

It has been suspected that calreticulin is also present in the nucleus of a cell (Opas *et al.*, 1991), and it has been shown to have a consensus nuclear localization sequence (Michalak, 1992) which is highly homologous to that of histone proteins. However, before this invention, its presence in the nucleus was unconfirmed and its function in the nucleus was unknown.

SUMMARY OF THE INVENTION

This invention relates to an isolated and purified product for use in modulating hormone responsiveness.

In one case, the product for modulating hormone responsiveness is calreticulin which inhibits hormone receptor induced gene transcription. In another case, the product is a mimetic of calreticulin. The product binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R.

In another case, the product for modulating hormone responsiveness is an antibody to calreticulin or a short peptide which binds to calreticulin. Such an antibody or peptide could promote hormone induced gene transcription by inhibiting calreticulin-hormone receptor interactions. The peptide may be one selected from a group consisting of: KGFFRR, KVFFKR, KAFFKR, KGFFKR, TGFFKR, KLGFFKR, KLDFFKR, KLGRFKR, KLGFFGR, KLGFFKG or modified derivatives of these peptides.

A product which reverses selectively calreticulin inhibitions of receptor binding to DNA response elements is part of this invention. One product which reverses selectively calreticulin inhibitions of retinoic acid to its DNA response elements is KLDFFKR. Another product which reverses selectively calreticulin inhibitions of androgen receptor binding to its DNA response elements is one selected from a group consisting of KLGFFGR and KLGFFKG. Other peptides may be designed by those skilled in the art to reverse selectively calreticulin inhibitions of other receptors binding to their DNA response elements.

The invention described in this patent application includes an isolated DNA molecule encoding an amino acid sequence for use in modulating hormone responsiveness. The isolated DNA molecule may encode the amino acid sequence for calreticulin. It may encode the amino acid sequence for part of a mimetic of calreticulin. It may encode a first amino acid sequence that binds to a second amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R.

The invention described in this patent application includes a method of treating a disease, disorder or abnormal physical state in a mammal by regulating hormone receptor induced gene transcription in a cell. The method could include regulating the activity, quantity or stability of a protein for use in hormone receptor induced gene transcription. The protein could be one that includes or binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R. One protein which binds to such sequence is calreticulin. The hormone receptor could be one selected from a group

consisting of: glucocorticoid receptor, minerolcorticoid receptor, androgen receptor, progesterone receptor, estrogen receptor, retinoic acid receptor, thyroid hormone receptor, vitamin D receptor and orphan receptors. The disease or disorder could be one selected from a group consisting of breast cancer, prostate cancer, promyelocytic leukemia, solid tumors, chronic inflammatory disease, such as arthritis and osteoporosis.

The method of treating the disease could include administering to the mammal a pharmaceutical comprising the protein, or an organic mimetic and a carrier. Another method of treating the disease could include administering to the mammal a pharmaceutical comprising an inhibitor of the protein and a carrier. A suitable carrier could be a lipid vesicle. As an alternative, the method could include decreasing or eliminating the quantity of calreticulin present in the cell; or decreasing the stability of calreticulin present in a cell.

The invention described in this patent application includes a kit containing a pharmaceutical comprising a protein for use in modulating hormone responsiveness together with a carrier. The protein included within the kit would be one that binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R. Such a protein would include calreticulin or a mimetic of calreticulin. The protein could be one that binds to calreticulin.

DEFINITIONS

In this application, the following terms have the following meanings, unless the context requires otherwise:

"A" means adenine

"Binds" means that under given conditions of ionic strength and temperature, a particular product binds to a substrate

"EDTA" means ethylenediaminetetraacetic acid

"EGF" means Epidermal growth factor

"ELISA" means enzyme-linked immunosorbent-assay

"F" means phenylalanine

"FGF" means Fibroblast growth factor

"G" means glysine

"HPLC" means high performance liquid chromatography

"IGF" means insulin-like growth factor

"K" means lysine

"KXFFYR" means an amino acid sequence, wherein X is G, A or V and wherein Y is K or R

"p60" means a 60 kDA protein, calreticulin

"PAGE" means polyacrylamide gel electrophoresis

"Peptide" includes amino acids, peptides, polypeptides and proteins

"R" means arginine

"RXR" means retinoid X receptor

"T" means Threonine

"TGF-B" means Transforming growth factor - β

"V" means valine

"VDR" means vitamin D receptor

"VDRE" means vitamin D response element

DESCRIPTION OF DRAWINGS

FIGURE 1A shows the isolation of p60 (calreticulin) from nuclei by affinity chromatography on KLGFFKR-sepharose.

FIGURE 1B shows immunofluorescent confocal images of TE-85 human osteosarcoma cell nuclei stained with an antibody against calreticulin.

FIGURE 2A shows that preincubation of purified p60 (calreticulin) with the recombinant receptor resulted in a dose-dependent inhibition in the formation of the complex between the receptor and the DNA.

2B shows that recombinant calreticulin inhibits the binding of the androgen-receptor to its response element.

2C shows that co-transfection of the calreticulin-containing plasmid resulted in a dose-dependent inhibition of chloramphenicol acetyltransferase activity induced by the androgen receptor.

FIGURE 3A shows that overexpression of calreticulin by calreticulin cDNA transfection in p19EC cells dramatically suppressed neuronal differentiation, as judged by the expression of a specific early marker of neuronal differentiation class III β-tubulin.

3B shows that overexpression of calreticulin by calreticulin cDNA transfection in p19EC cells dramatically suppressed neuronal differentiation, as judged by the expression of a specific early marker of neuronal differentiation class III β-tubulin.

3C shows the modulation of neuronal differentiation of P19EC cells by different levels of expression of calreticulin: (D) shows the increased levels of calreticulin inhibit neuronal differentiation. (F) shows the decreased levels of calreticulin enhance neuronal differentiation.

FIGURE 4 Calreticulin interacts with the VDR protein to inhibit the binding of VDR homodimers and VDR-RXR heterodimers to characterized VDREs.

FIGURE 4A VDR and RXRb were in vitro translated and incubated with a labeled oligonucleotide corresponding to the murine osteopontin VDRE in the presence or absence of purified calreticulin.

FIGURE 4B Baculovirus-expressed VDR and RXRa were incubated with a murine osteocalcin VDRE oligonucleotide probe. Increasing amounts of purified calreticulin were added. The binding reactions were analyzed using the gel retardation assay on a 7% non-denaturing gel.

FIGURE 4C SDS-PAGE analysis of immunoprecipitation reactions.

FIGURE 5 Expression pattern of calreticulin and osteoblast phenotype markers during MC3T3-E1 osteoblastic differentiation.

FIGURE 5A Ethidium bromide stained ribosomal RNA showing equivalent loading for all samples.

FIGURE 5B The RNA was hybridized to specific probes for calreticulin.

FIGURE 5C Same as 5B, but with probes for osteopontin.

FIGURE 5D Same as 5B, but with probes for osteocalcin.

FIGURE 6 Expression of the calreticulin protein in parental cells, overexpressing clones, and control clones.

FIGURE 7 Calreticulin overexpression affects the morphology of osteoblastic cells at confluence.

FIGURE 8 Calreticulin overexpression selectively inhibits the vitamin D-induced stimulation of osteocalcin expression without affecting vitamin D-stimulated osteopontin expression.

FIGURE 9 Constitutive calreticulin expression inhibits mineralization.

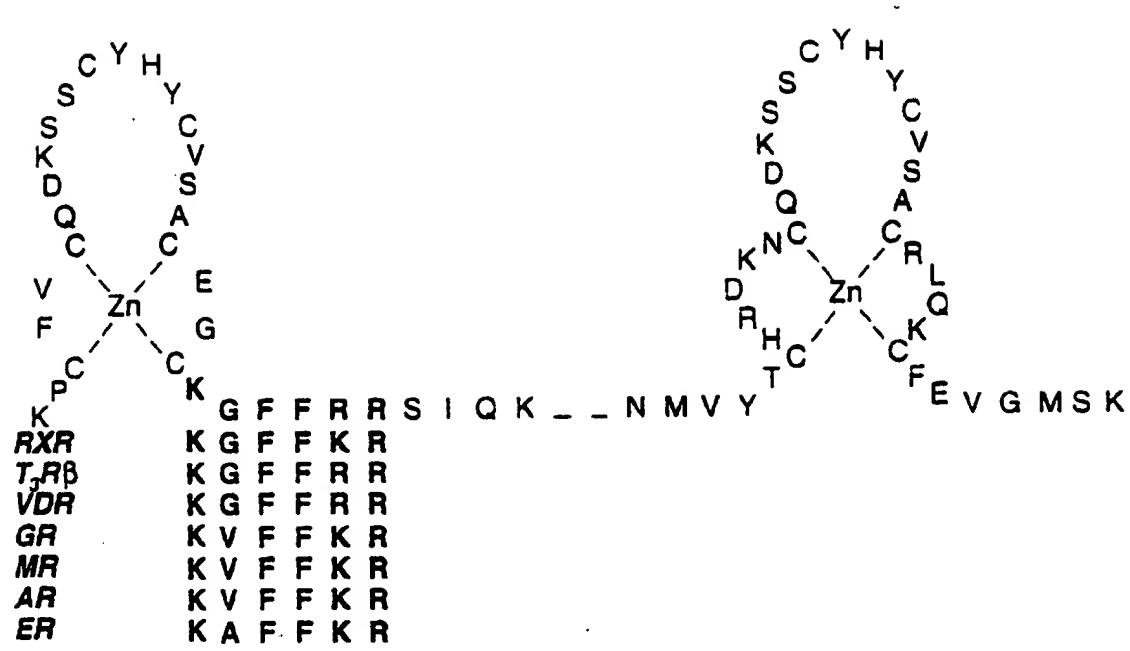
FIGURE 10 Calreticulin overexpression inhibits the vitamin D-induced stimulation of calcium incorporation into the extracellular matrix.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A highly homologous amino acid sequence, KXFFYR (where X is either G, A or V and where Y is either K or R), has been found to be present in the DNA binding domain of all known members of the steroid hormone receptor family (Fuller, 1991), and amino acids in this sequence make direct contact with nucleotides in their DNA responsive elements, and are crucial for DNA binding (Luisi, 1991).

Naturally occurring and recombinant calreticulin, inhibit the binding of receptors to DNA. Thus, calreticulin and proteins which mimic or bind to calreticulin modulate nuclear hormone receptor regulation of gene transcription.

By way of example, the amino acid sequence of the DNA binding domain of RAR is set out below:



X G F F K R : Calreticulin Binding Sequence in Integrin α Subunits.

Calreticulin binds to nuclear hormone receptors by interacting with the amino acid sequence KXFFYR. The interaction results in a profound inhibition of nuclear hormone receptor DNA binding activity which can be reversed by soluble competing synthetic peptides with the generic sequence KXFFYR . The inhibition of DNA binding by calreticulin can also be reversed by an antibody to or inhibitor of calreticulin. Transient or stable overexpression of calreticulin by cDNA transfection also results in the inhibition of nuclear hormone receptor induced gene transcriptional activity. Furthermore, decreased expression of calreticulin by stable transfection of antisense calreticulin cDNA results in increased sensitivity of the cells to hormones due to the increased transcriptional activity of the nuclear hormone receptor.

Hence, a proportion of nuclear hormone receptors may be occupied by calreticulin in a constitutive manner, and decreased regulation of expression of calreticulin may therefore result in an effective increase in the number of

unoccupied receptors leading to increased transcriptional activity of these receptors.

By this invention, hormonal sensitivity can be manipulated by (i) increasing or decreasing the intracellular concentration of calreticulin, or (ii) by inhibiting the interaction of calreticulin with nuclear hormone receptors by peptides, peptide mimetics, and antibodies against calreticulin or the KXFFYR sequence.

The nuclear hormone receptors that interact with calreticulin include androgen receptor, retinoic acid receptors (RAR and RXR), glucocorticoid receptor, and the vitamin D receptor. In all of these cases, calreticulin inhibits receptor binding to DNA, and overexpression of calreticulin results in an inhibition of receptor mediated transcriptional activity. In the case of the retinoic acid receptor system, the decreased regulation of expression of calreticulin results in an increased sensitivity of the cells to differentiation by retinoic acid.

Except whether otherwise indicated in the following examples, cell lines were obtained from American Type Culture Collection - ATCC; Chemical reagents were purchased from Sigma Chemicals, St. Louis, MO; BioRad, Richmond, CA; and Amersham Corp., Oakville, ON; Radioisotopes were purchased from Amersham Corp., Oakville, ON; Peptides were synthesized by HSC/Pharmacia Biotechnology Service and Department of Clinical Biochemistry, University of Toronto; Oligonucleotides were synthesized by University of Toronto - Carbohydrate Research Group; Centrifuges used were from Beckman or Eppendorf.

Example 1 Calreticulin is Present in Nucleus of Cells

The conservation of the KXGFFYR sequence in the α-subunits of integrins is shown in Table I.

A computer search of the Swiss protein data bank for the presence of this sequence motif in other proteins revealed that a highly homologous sequence is present in the DNA binding domain of all members of the nuclear hormone receptors (Table 1) (Fuller, 1991; Carson-Jurica,*et al.*, 1990). Because amino acids in this motif have been demonstrated to be essential for the binding of nuclear hormone receptors to their DNA responsive elements

Table I

Conservation of an Amino Acid Sequence Motif in the Integrin Alpha-subunit Cytoplasmic Domains and in the Steroid Hormone Receptor Family

Integrins*		Steroid Nuclear Receptors	
a1	**K I G F F K R**	RARa	A C E G C **KGFFRR** S I Q K
a2	**K L G F F K R**	T_3Rb	T C E G C **KGFFRR** T I Q K
a3	**K C G F F K R**	VDR	T C E G C **KGFFRR** S M K R
a4	**K A G F F K R**	GR	T C G S C **KVFFKR** A V E G
a5	**K L G F F K R**	MR	T C G S C **KVFFKR** A V E G
a6 (A)	**K C G F F K R**	AR	T C G S C **KVFFKR** A A A G
a6 (B)	**K C G F F K R**	PR	T C G S C **KVFFKR** A M E G
a7	**K L G F F K R**	ER	S C E G C **KAFFKR** S I Q G
a8 (chick)	**K C G F F D R**	RXR	S C E G C **KGFFKR** T V R K
av	**R M G F F K R**	Steroid receptor TR2	T C E G C **TGFFKR** S I R K
Mac-1	**K L G F F K R**	Nerve growth factor induced protein 1-B	T C E G C **KGFFKR** T V Q K
p150	**K V G F F K R**	Early response protein NAK1	T C E G C **KGFFKR** T V Q K
PS2 (Drosophila)	**K C G F F N R**	Chorion Factor I	S C E G C **KGFFKR** T V R K

(Luisi *et al.*, 1991; Haird *et al.*, 1990), we wanted to determine whether a 60 kDa protein isolated by affinity chromatography on a KLGFFKR-sepharose affinity matrix (Rojiani *et al.*, 1991) could modulate DNA binding and transcriptional activities of nuclear hormone receptors.

In Table I, the sequences indicated with an asterisk were obtained as described in Rojiani *et al*, 1991. GR: Glucorticoid receptor; MR: Minerolcorticoid receptor; AR: Androgen receptor; PR: Progesterone receptor; ER: Estrogen receptor.

Although calreticulin contains a KDEL motif at its C-terminus and is therefore thought to be resident in the endoplasmic reticulum (McCauliffe *et al.*, 1990; Fliegel *et al.*, 1989; Michalak *et al.*, 1992), it also has a nuclear targeting signal (McCauliffe *et al.*, 1990; Michalak *et al.*, 1992; Marzluff *et al.*, 1985), raising the possibility that this protein is also present in the nucleus (Michalak *et al.*, 1992). The presence of p60 in nuclei was demonstrated by affinity chromatography of human osteosarcoma cell (HOS) nuclear extracts on a KLGFFKR-affinity column (FIGURE 1).

Nuclei were purified from HOS cells by established methods (Luisi *et al.*, 1991). The purified nuclei were either lysed in PBS containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and 1 mM PMSF, or were applied to a glass coverslip and stained for nuclear antigen with anti-nuclear monoclonal antibody MAB1218 obtained from Chemicon Int. Inc., Tamecula, CA. The nuclei were visualized by indirect immunofluoresence. The total cellular or nuclear extracts were subjected to affinity chromatography on a KLGFFKR-affinity matrix, and the p60 isolated (Rojiani *et al.*, 1991).

Cell extracts were prepared from whole cells or from purified nuclei and applied to KLGFFKR-sepharose affinity matrix. Bound proteins were eluted with EDTA and analyzed by SDS-polyacrylamide gel electrophoresis (Rojiani *et al.*, 1991). The separated proteins were electrophoretically transferred to nitrocellulose filters and probed with an anti-calreticulin antibody. In FIGURE 1, Lane 1: Total cellular extract; Lane 2: EDTA eluted material from affinity column to which total cellular extract was applied; Lane 3: Nuclear extract; Lane 4: EDTA eluted

material from affinity column to which nuclear extract was applied. Arrow indicates the position of p60.

Indirect immunofluoresence of HOS cells or purified nuclei with anti-calreticulin antibody also demonstrated intranuclear calreticulin expression, as shown in FIGURE 1B. Confocal microscopy was carried out using a BioRad MRC 500 system. Note the non-nucleolar, intranuclear staining of the cell in (a) and (b), and the complete exclusion of intranuclear staining in the cell in (c). These data suggest that the expression of calreticulin in the nucleus is a regulated process.

These results confirmed the presence of a calreticulin-related p60 protein in nuclei.

Example 2 The Sequence KXFFYR is Present in All Known Members of the Nuclear Receptor Family

As shown in Table I, the sequence KXFFYR is present in all known members of the nuclear receptor family. The region containing this sequence in the DNA-binding domains of these receptors has been shown to play a crucial role in DNA sequence recognition (Luisi *et al.*, 1991). Thus calreticulin, by binding to this common sequence, modulates the binding of all members of the receptor family to DNA. By way of example, we have demonstrated the inhibition by calreticulin of the interaction of the androgen receptor with its DNA response element (see Examples 3 and 4) and of the retinoic acid receptor heterodimer complex (RAR/RXR) with its DNA response element (see Example 5).

Example 3 Ability of Calreticulin to Modulate Binding of Nuclear Hormone Receptors *In Vitro*

To determine whether p60 (calreticulin) could directly modulate the binding of nuclear hormone receptors to DNA via the KXFFYR sequence, the interaction of the DNA binding domain of recombinant androgen receptor with its hormone responsive element was analyzed by carrying out gel mobility shift assays.

As described in Rennie *et al.*, 1993, DNA binding domain of recombinant rat androgen receptor was prepared as a GST-fusion protein using the pGEX-

3X vector and purified by glutathione-agarose affinity chromatography. p60 (calreticulin) was purified by affinity chromatography on KLGFFKR-sepharose, followed by gel electrophoresis as described in detail in Rojiani *et al.*, 1991. Purified AR and p60 (calreticulin) were found to be greater than 90% and 95% pure, respectively, as determined by SDS-PAGE and Coomassie Blue staining. Recombinant calreticulin (GST-fusion protein) was prepared as described by Baksh and Michalak, 1991. Gel retardation assays were carried out as described by Rennie *et al.*, 1993. To analyze the effect of p60, or recombinant calreticulin, on receptor-DNA binding activity, the AR was pre-incubated with p60 for 30 min at 4°C. To analyze the effects of synthetic peptides, anti-calreticulin antibody, and non-immune IgG on p60 inhibition of AR-ARE binding, the peptide, antibody or IgG were pre-incubated with p60 for 30 min at 4°C. The androgen receptor preparation was then added to these mixtures and further incubated for 30 min at 4°C.

Affinity purified DNA binding domain of the recombinant rat androgen receptor (AR) was pre-incubated with or without the indicated concentrations of purified p60 at 4°C for 30 min. After this pre-incubation the reaction mixtures were incubated with ^{32}P-labeled 26 base pair ARE (Rennie *et al.*, 1993) (androgen response element), and analyzed by gel retardation assay. The sequence of the ARE used was:

5′ GTAAAGTACTCCAAGAACCTATTTgt 3′

3′ CATTTCATGAGGTTCTTGGATAAAca 5′

In FIGURE 2A, the following lanes show the following results: Lane 1 ^{32}P-labeled ARE by itself; Lane 2: Retardation of ARE by AR; Lane 3: Effect of pre-incubation of 0.11 µg of purified p60 with AR on AR-ARE binding; Lane 4: Effect of the addition of a 25-fold molar excess of KLGFFKR synthetic peptide to p60 on AR-ARE binding. Lanes 3, 5, 7, & 9: Effect of increasing concentrations of p60 (from 0.11 µg to 0.33 µg) on AR-ARE binding; Lanes 4, 6, 8, & 10: Reversal of p60 inhibition of AR-ARE binding by KLGFFKR peptide. Lane 11: Effect of addition of anti-calreticulin antibody to p60 inhibition of AR-ARE binding. Lanes 12-15: Increasing amounts of p60 in the presence of anti-

calreticulin antibody. Increasing the p60 concentration overcomes the effect of antibody on p60 inhibition of AR-ARE.

In FIGURE 2B, the following lanes show the following results: Lane 1: ^{32}P-labelled ARE alone; Lane 2: Retardation of ARE by AR in the presence of gluthathione-S-transferase (GST); Lane 3: Inhibition by GST-calreticulin (GST-calreticulin) of AR-ARE interaction; Lanes 4 and 5: Reversal of this inhibition by KLGFFKR peptide; Lanes 6 and 7: Inability of the scrambled peptide (KLRFGFK) in reversing the effect of calreticulin on AR-ARE interaction; Lanes 8 and 9: The peptide KVFFKR can also reverse the inhibition by calreticulin of the AR-ARE interaction. The concentration of calreticulin used was 2 µg and the peptides were used at a 50-fold molar excess concentration.

As shown in FIGURE 2, the migration of a ^{32}P-labeled 26 base pair DNA androgen responsive element residing at positions -115 to -140 of the rat probasin gene promoter (Rennie *et al.*, 1993) was retarded by the androgen receptor DNA-binding domain; indicating the formation of a complex between the receptor and the DNA (Rennie *et al.*, 1993). Pre-incubation of purified p60 (calreticulin) with the recombinant receptor resulted in a dose-dependent inhibition in the formation of this complex (FIGURE 2A, lanes 3, 5, 7, & 9). The sequence specificity of this inhibition was demonstrated by the finding that the inhibition by p60 (calreticulin) of receptor-DNA binding was reversed by the addition of competing KLGFFKR peptide (FIGURE 2A, lanes 4, 6, 8, & 10) or KVFFKR (FIGURE 2B, lanes 8 and 9), whereas a scrambled peptide (KLRFGFK) was much less effective (FIGURE 2B, lanes 6 and 7). An antibody to calreticulin, which cross-reacts with p60, also reversed this inhibition by p60 (FIGURE 2A, lane 11), demonstrating p60 specificity. Non-immune IgG did not have any effect on the inhibition of receptor-DNA interaction by p60 (FIGURE 2A, lane 15). Furthermore, neither KLGFFKR peptide, anti-calreticulin antibody, nor non-immune IgG by themselves had any effect on the receptor-DNA interaction (data not shown). p60 did not effect the binding of AP-1 to DNA, and other proteins of similar size (e.g. bovine serum albumin) also did not have any effect on the nuclear receptor-DNA interaction (data not shown).

Recombinant calreticulin (obtained from Dr. Michalak, Edmonton, Alta) (Baksh *et al.*, 1991), in the form of a GST-fusion protein, also inhibited the binding of the androgen-receptor to its response element (FIGURE 2B, lane 2), and this inhibition was also reversed by KVFFKR peptide, (FIGURE 2B, lane 2), but not by a scrambled peptide KLRFGFK (FIGURE 2B, lane 1) confirming that the p60 purified on the KLGFFKR affinity matrix and calreticulin are functionally similar in terms of binding to nuclear hormone receptors, and that a synthetic peptide, KVFFKR can competitively inhibit the binding of calreticulin to the KVFFKR sequence of the androgen receptor.

Example 4 Inhibition of Transcriptional Activity of the Androgen Receptor *In Vivo*.

To determine whether calreticulin also inhibited the transcriptional activity of the androgen receptor *in vivo*, expression vectors containing full-length calreticulin (McCauliffe *et al.*, 1990) and androgen receptor (Rennie *et al.*, 1993), cDNAs were co-transfected into Vero fibroblasts together with a chloramphenicol acetyl transferase (CAT) reporter plasmid driven by the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). MMTV-LTR contains androgen response elements (Rennie *et al.*, 1993).

FIGURE 2C shows inhibition of androgen-induced CAT activity by calreticulin. Vero fibroblasts were cotransfected with an MMTV-CAT reporter vector and various amounts of a calreticulin expression vector and the pRC-CMV vector alone (Invitrogen) using the calcium phosphate method (Filmus *et al.*, 1992). In all transfections 10 µg of a β-galactosidase expression vector and 10 µg of an androgen receptor expression vector (Seed *et al.*, 1988) were included. Transfected cells were incubated in medium alone or in the presence of 100 nM R1881 (synthetic androgen) for 12 h. Cells were then lysed and CAT activity measured (Seed *et al.*, 1988). An aliquot of the cell extracts was also assayed for β-galactosidase activity. This activity was used to standardize the measurement of CAT levels in each experiment by taking into account the efficiency of the transfection. Every sample was tested in quadruplicate and the average activity calculated. CAT activity induction as defined as the ratio between the standardized CAT activity of the R1881 treated cells and the

corresponding untreated cultures. The Vero cells were grown in a-minimum essential medium containing 10% charcoal-treated calf serum.

As shown in FIGURE 2C, co-transfection of the calreticulin containing plasmid resulted in a dose dependent inhibition of CAT activity induced by the androgen receptor. Furthermore, immunoprecipitation of calreticulin from ^{35}S-methionine/cysteine labeled, androgen receptor transfected Vero cells, resulted in the co-precipitation of the 110 kDa androgen receptor, indicating a direct interaction between calreticulin and the androgen receptor (S. Dedhar and C. Leung-Hagesteijn, unpublished observations).

These data demonstrate that not only can calreticulin bind to the androgen receptor DNA binding domain and inhibit its interaction with the androgen response elements *in vitro*, it can also inhibit the transcriptional activity of the androgen receptor *in vivo*. Although other 59 kDa proteins have been found in complexes with several steroid hormone receptors (Lebeau *et al.*, 1992; Tai *et al.*, 1992), they are distinct from calreticulin, and none of them have an effect on binding of the receptors to their DNA responsive elements.

Example 5 Regulation by Calreticulin of Hormone Receptor Induced Gene Transcription

In order to demonstrate a physiological significance of the finding that calreticulin can bind to the DNA binding domain of nuclear hormone receptors and modulate their transcriptional activity, we utilized a retinoic acid responsive system i.e. the induction of neuronal differentiation by retinoic acid in P19 embryonal carcinoma cells (McBurney *et al.*, 1982). We predicted that increased expression of calreticulin would suppress retinoic acid induced neuronal differentiation, whereas decreased expression would result in the release of calreticulin inhibition, and allow for a more rapid rate of neuronal differentiation.

The full length 1.9 Kb calreticulin cDNA (McCauliffe *et al.*, 1990) was obtained from Dr. R.D. Sontheimer, Texas and was subcloned into pRC/CMV (Invitrogen, San Diego, CA) expression vector in the sense and antisense orientation. pRC/CMV, pRC/CMV-Cal-1 (sense), or pRC/CMV-Cal-2 (antisense) expression plasmids were then transfected into P19 embryonal

carcinoma cells by electroporation. Neomycin-resistant transfectant cells were then selected by growth in the presence of 600 µg/ml G418 and the resistant cells were maintained in 100 µg/ml G-418. Cal-1 and Cal-2 transfectants were subcloned by limiting dilution, and the subclones were screened for calreticulin expression by Western blot analysis of cell lysates with an anti-calreticulin antibody (Rojiani *et al.*, 1991).

Retinoic acid neuronal differentiation was induced as described previously (McBurney *et al.*, 1982; Dedhar *et al.*, 1991) and class III β-tubulin expression was analyzed by Western blotting with a class III β-tubulin monoclonal antibody (TuJ1). This antibody was obtained from Dr. A. Frankfurter, University of Virginia, Charlottesville, VA, USA. The bRARE-luciferase transient transfections in p19 (Neo), Cal-1 and Cal-2 cells were carried out as described in Tini *et al.*, 1993. The vector bRARE(3) tk-LUC was constructed by linking 3 copies of the 32 base pair sequence that defines the RARE upstream from the RAR-b gene (de The, *et al.*, 1990; Sucov *et al.*, 1990) to the minimal thymidine kinase promoter and the firefly luciferase gene.

The level of calreticulin expression was estimated by Western blot analysis of cellular lysates (Rojiani *et al.*, 1991) followed by densitometric scanning. For Northern blot analysis total cellular RNA (15 µg) from the indicated cell lines was hybridized to ^{32}-P-labeled CRABP(II) cDNA (Giguere *et al.*, 1990) at 65°C using Rapid Hyb buffer (Amersham Corp.). The blot was stripped and reprobed with a mouse actin cDNA probe to check for equal loading of RNA. Values for relative mRNA levels were derived from quantitation of the signal in each lane using a Molecular Dynamics Phosphorimager. CRABPII mRNA levels were normalized against the corresponding actin mRNA signal.

The level of expression of calreticulin was modulated in P19 EC cells by transfection with calreticulin cDNA inserted in the sense or antisense orientation in the pRC/CMV (Invitrogen Corp., San Diego, CA) expression vector. P19 EC cell subclones overexpressing calreticulin (Cal-1), or anti-sense transfectants with reduced calreticulin expression (Cal-2), as well as control transfected cells (Neo), were subjected to induction of neuronal differentiation by retinoic acid as

described previously (McBurney *et al.*, 1982; Dedhar *et al.*, 1991). The expression of neuron-specific class III β-tubulin (Lee *et al.*, 1990; Alexander *et al.*, 1991) was then analyzed 48 hr (A) or 72 hr (B) after the addition of all-trans retinoic acid (5 mM). Cal-1 (1A2 and 1D2) clones were transfected with pRC/CMV containing calreticulin cDNA in the sense orientation. Cal-2 (1A4 and 1B4) clones were transfected with pRC/CMV containing calreticulin cDNA in the anti-sense orientation. (C): Effect of levels of calreticulin expression on retinoic acid mediated neuronal differentiation.

Cells were stained with anti-class III β tubulin antibody (TuJi) followed by FITC conjugated secondary antibody as described above. A and B: P19 (neo) EC cells; C and D: P19-Cal-1 EC cells; E and F: P19 Cal-2 EC cells. A, C and E; untreated cells. B, D and F: 6 days at RA (0.5 μM) treated cells. The cells were visualized using a Zeiss Axioscop microscope under oil immersion and photographed with Kodak T-Max 400 film. Magnification 100 X.

As shown in FIGURE 3A and B, overexpression of calreticulin (Cal-1), by calreticulin cDNA transfection in P19 EC cells indeed dramatically suppressed neuronal differentiation, as judged by the expression of a specific early marker of neuronal differentiation, class III β-tubulin (Lee *et al.*, 1990; Alexander *et al.*, 1991). In contrast, decreased expression of calreticulin (Cal-2), by anti-sense calreticulin cDNA transfection, resulted in markedly enhanced expression of class III β-tubulin.

FIGURE 3C clearly shows the inhibition of neuronal differentiation by calreticulin overexpression and enhanced differentiation by diminished calreticulin expression.

The effect of calreticulin levels on retinoic acid induced neuronal differentiation occurs via the direct regulation of retinoic acid responsive genes, as demonstrated by an inverse relationship between calreticulin expression level and RARE-driven luciferase gene expression (Dedhar *et al.*, 1994). Furthermore, the endogenous regulation of expression of the retinoic acid responsive genes, CRABPII (Giguere *et al.*, 1990) and RAR-b (de The, *et al.*, 1990; Sucov *et al.*, 1990) are substantially decreased in Cal-1 transfectants, but

are either unchanged or slightly increased in the calreticulin-antisense Cal-2 transfectants (Dedhar *et al.*, 1994).

Collectively, these results demonstrate that calreticulin, by binding to the conserved KXFFYR sequence in the DNA binding domain of nuclear hormone receptors (Table I), can modulate gene expression and cellular phenotypes, such as cell differentiation. Calreticulin may also behave as a signal modifier by translocating between the nucleus and the cytoplasm, where it has been shown to bind, via an identical sequence motif, to the intracellular domains of the α-subunits of integrin receptors (Rojiani *et al.*, 1991).

Example 6 KLGFFKR Modulates Retinoic Acid Induced Gene Transcription *In Vivo*

In order to test whether a peptide based on the calreticulin-binding KXGFFKR sequence modulates retinoic acid induced gene transcription in live cells, the p19 cells were transfected with a reporter vector consisting of a retinoic acid response element fused to the luciferase gene. Since these cells contain endogenous retinoic acid receptors RAR and RXR, treatment with retinoic acid resulted in an induction of the RARE driven luciferase activity (see Table II).

Cell culture conditions: Mouse embryonic carcinoma (P19) cell, grown in 60 mm dishes in 7.5% donor calf serum, 2.5% fetal calf serum alpha MEM (Gibco/BRL) were treated with the peptides KLGFFKR or KLRFGFK for three hours or overnight for 20 hours at 37°C, 5% CO_2. With few exceptions, KLXFFKR is the peptide sequence specific within the binding domain of all steroid receptors. KLRFGFK is the scrambled peptide of the above sequence. Subsequently each plate was washed four times with serum free alpha MEM to remove excess peptides and replenished with fresh serum containing media. Cells were then transfected by standard calcium phosphate precipitation method (*Current Protocols in Molecular Biology* 9:1) with 1 microgram bRARE in pTKluc, 1.5 microgram pRSV bgal, 3 microgram pKS (carrier) per 60 mm dish. Following a 16 hour incubation at 37°C 5% CO_2, each plate was washed two times with serum free alphaMEM and replenished with serum containing media supplement with 10^7 M retinoic acid (Sigma R2625) and 800

microgram/ml G418 (Gibco 1181-031). Following another 24 hours incubation cells were washed three times with PBS and each 60 mm dish of cells was lysed in 100 microliters of 1% triton X100, 100 mM KPO_4 pH7.8, 1 mM DTT. Cell lysates were stored at -70°C. Prior to luciferase/bgal assays, cell debris were spun out on an Eppendorf microfuge (5415C) at 4°C full speed for 20 minutes.

Luciferase assay: All reagents were equilibrated to room temperature and each sample was assayed independently. Ten microliters of cell lysate was incubated with 50 microliters of luciferase reagent (Promega E1483) in an Eppendorf tube. Thirty seconds later, the sample was immediately counted in a Beckman scintillation counter (LS6000IC) for one minute using an open window. Standards in the range of 0.001 nanogram to 1.0 nanogram was used to establish the linearity of the assay.

Beta-gal assay: Assay was done in a microtitre plate (Linbro 76-232-05). Ten microliters of cell lysate were mixed with 90 microliters of bgal reagent in 88 mM phosphate buffer, 11 mM KCl, 1 mM $MgCl_2$, 55 mM 2ME, 4.4 mM chlorophenol red b-D-galactopyranoside (BMC 884-308). Incubation period varied from 30 minutes to 2 hours at 37°C. Results were read at 570 nm in an ELISA reader (Dynatech MR5000).

The pre-incubation of these cells with the specific KLGFFKR peptide resulted in a dose-dependent increase in luciferase activity indicating a stimulation of the retinoic acid receptor mediated gene transcription (Table II). The pre-incubation of a control, scrambled peptide had no such effect. The transfection efficiency was controlled by co-transfection of the β-galactiosidase gene and the subsequent measurement of β-galactosidase activity as described above.

Similarly, a peptide which inhibited calreticulin-androgen receptor interaction modulated the differentiation state of prostate cancer calles (LnCap cell line) (Table III). The differentiation marker used was PSA (prostatic specific antigen). The peptide alone, when added to those cells, induced PSA which was normally induced by androgens. When added together with a synthetic

androgen (R1881) the peptide enhanced PSA expression. The experiment showed the feasibility of the peptide based therapeutic approach.

These results show that peptides based on the KXFFYR sequence can be used to modulate hormone responsiveness by influencing the binding of calreticulin to the hormone receptors in live cells. Thus in the experiments described, peptides were able to effectively compete for calreticulin binding with the KGFFRR sequence in the retinoic acid or androgen receptor. This activated the calreticulin-bound receptors resulting in increased transcriptional activity.

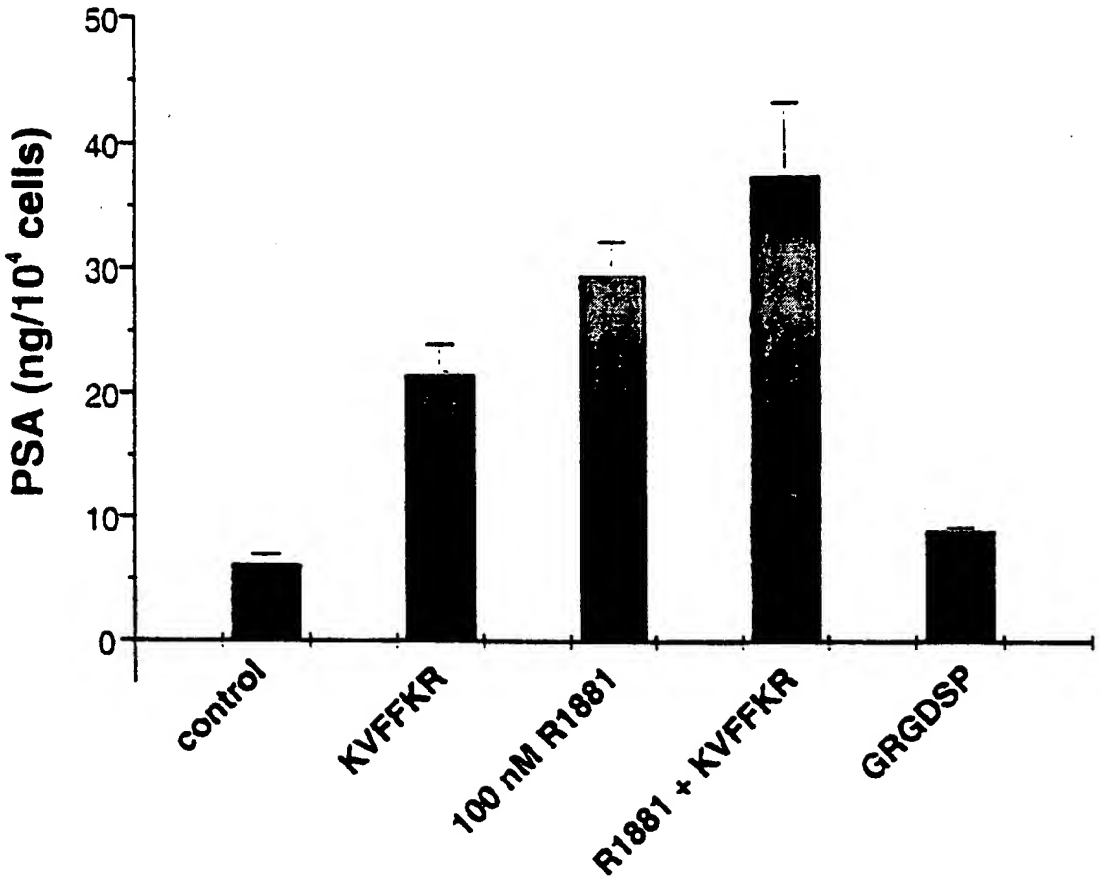
Table II

OVERNIGHT INCUBATIONS

Preincubations	**Concentrations micromolar**	**Luciferase x 10^6**	**Beta-gal**	**Corrected values luc/bgal**
KLGFFKR	10	13	0.395	32.91
KLGFFKR	50	20	0.443	45.15
KLGFFKR	100	28	0.452	61.95
KLRFGFK	10	14	0.333	42.04
KLRFGFK	50	16	0.387	41.34
KLRFGFK	100	14	0.434	32.26
Controls				
no retinoic acid	0	0.17	0.348	0.49
10^{-7} retinoic acid	0	8.3	0.323	25.7

Table III

EFFECTS OF PEPTIDES ON PSA SECRETION
BY LNCAP CELLS IN VITRO



Modulation of PSA secretion by androgen responsive LnCap cells

The cells were treated either with tissue culture medium (TCM) alone, TCM containing the synthetic androgen, R1881 or the androgen receptor derived peptide KVFFKR, or both. After overnight incubations, PSA was measured in conditioned medium by radioimmunoassay.

Example 7 Peptides Having Differential Specificities for Disrupting Different Hormone Receptor-Calreticulin Interactions

To identify such peptides, we utilized gel mobility shift assays (see Example 3) in which known concentrations of purified recombinant androgen receptor, estrogen receptor, retinoic acid receptors (RAR/RXR and RXR/RXR)

(Shago *et al.*, 1994) and vitamin D receptor (Xu *et al.*, 1993) were incubated with known concentrations of either recombinant calreticulin, or calreticulin purified by affinity chromatography on a KLGFFKR affinity column (see Example 3) in the presence of the respective ^{32}P-labelled DNA response elements and known concentrations of synthetic peptides based on the KXFFYR sequence. In addition to the linear peptides, some peptides were cyclized by adding cysteine residues at either ends. These experiments resulted in the identification of peptides which have distinct antagonistic specificities for the interaction of different hormone receptors with calreticulin.

In order to derive peptides which might be specific for one receptor over another one, we have undertaken the synthesis of a series of peptides listed in Table IV.

These peptides were tested in gel mobility shift assays (described in Example 3) using equivalent concentrations of various receptors: retinoic acid receptors (RAR/RXR), vitamin D receptor (VDR), estrogen receptor (ER), androgen receptor (AR) and glucocorticoid receptor (GR), and their respective DNA response elements. These experiments identified specific peptides for use against individual receptors.

Experiments using the RAR/RXR, RXR/RXR or VDR/RXR receptors indicated that the KLGFFKR peptide was 10-fold more potent against the VDR/RXR heterodimer compared to RAR/RXR heterodimer, and was 4-fold more potent against the RXR/RXR heterodimer compared to the RAR/RXR receptor.

Our data have also identified the amino acids within this sequence which are crucial for activity as shown in Table V. Table V shows that certain peptides exhibited selectivity for inhibiting calreticulin-receptor interaction in a receptor specific manner. This Table relates to the retinoic acid receptors (RAR/RXR) and the androgen receptor (AR).

Table IV

Proposed Peptides

Proposed Peptides	Variants	Exploring Protection	Exploring Hydrophobic Patch
RKFFGK	Reversed	X	
d(CKGFFKR)	D-amino acid version	X	
FGKKRK	another scrambled peptide	X	
Ac-KGFFKR	Acetylated peptide	X	
KGLFKR			X
KGFLKR			X
KGYFKR			X
KGFYKR			X
KGPFKR			X
KGFPKR			X
KFGFKR	Inversion		X
KGDFKR			X
KGFKDR			X

Table V

Relative Ability of Peptides to Reverse Calreticulin Inhibitions of Retinoic Acid Receptor (RAR/RXR) and Androgen Receptor (AR) Binding to Respective DNA Response Elements
Response Elements

Peptide	% Reversal RAR/RXR	AR
KLGFFKR	100%	62%
KGFFKR	100%	N.D.
KVFFKR	N.D.	100%
KLDFFKR	73%*	0%*
KLGRFKR	24%	10%
KLGFRKR	20%	6%
KLGFFGR	65%	85%*
KLGFFKG	22%*	63%*

These data were obtained utilizing Gel mobility shift assays as described previously (Nature 367: 480-483, 1994). The data were quantified using a Phosphorimager (Molecular Dynamics).

* significant selectivity of these peptides for one receptor over the other. The most critical amino acids appear to be F, F, and R in the sequence KLGFFKR. These three amino acids are completely invariant in all steroid and nuclear hormone receptors as well as in integrins.

Thus using the RAR/RXR system in gel mobility shift assays, the two phenyalanines, as well as the terminal arginine, were found to be absolutely essential, since substitution of these resulted in the abrogation of the peptide activity (Table V).

The peptides identified from these gel mobility shift assays are being used in cellular assays described below.

Retinoic acid-receptor specific peptides: These are tested in the P19 retinoic acid induced neuronal differentiation assay described in Example 5.

Vitamin D-receptor specific peptides: These are tested in the MC3T3-E1 osteoblastic cells which can be induced to differentiate into

osteoblasts and form a calcified matrix (mineralize) with vitamin D. The ability to mineralize by monitoring ^{45}Ca incorporation is determined after treatment with peptides.

Estrogen-receptor specific peptides: These are tested for ability to modulate estrogen-responsive breast cancer cell line proliferation. ER positive cells, e.g. MCF7 and T47-D are used.

Androgen-receptor specific peptides: These were tested in prostate carcinoma LnCAP cells which are androgen responsive and express the androgen-receptor.

Glucocorticoid-receptor specific assays: These are tested in dexamethasone treated peripheral blood lymphocytes.

Example 8 Regulation of Endogenous Level of Expression of Calreticulin

In murine P19 embryonal carcinoma cells, overexpression of calreticulin inhibits all-trans retinoic acid responsiveness, whereas downregulating calreticulin by antisense cDNA transfection results in an enhancement of retinoic acid response (see Example 5). In order to determine whether such modulation of calreticulin expression results in changes in the responsiveness to other steroid hormones and vitamins, the PRC-CMV based calreticulin vectors CAL-1 (sense cDNA) and CAL-2 (antisense cDNA) are used to stably transfect mouse osteoblastic cells (MC3T3 E1), chicken osteoclast precursors, normal rat mammary epithelial cells (Darcy *et al.*, 1991) and chemically transformed rat mammary adenocarcinoma cells (ATCC CRL1743), as well as estrogen and progesterone responsive human breast carcinoma cells (MCF-7 and T47-D).

Calreticulin expression levels in these cell types are determined at the outset by Western blot analysis. In addition to utilizing these stable expression vectors, we construct inducible calreticulin expression sense- and antisense-cDNA expression vectors driven by strong metal inducible promoters (Filmus *et al.*, 1992). The inducible vectors allow us to turn calreticulin expression on or off at will. In the MC3T3 cells, 1,25 dihydroxyvitamin D3 has a proliferative effect on these cells at subconfluency, but when added to confluent,

mineralizing cultures, it enhances the mineralization process. The transfected cells are analyzed for the level of calreticulin expression by Western blot analysis as described by us previously as well as by Northern blot analysis for mRNA levels. The effect of up or down regulation of calreticulin is determined in terms of the above mentioned responses to 1,25 dihydroxyvitamin D3. In addition, the effect on the expression of vitamin D responsive genes, such as c-*fos* and integrin b3 subunit (Xu *et al.*, 1993) is determined by Northern blot and Western blot analysis. These cells are transfected with a reporter construct consisting of a vitamin D response element (VDRE) driving the luciferase gene. The luciferase activity in mock transfected versus calreticulin sense- and antisense-cDNA transfected cells (stable and inducible) is then be determined as described by us previously (see Example 6).

Similar experiments are carried out in chicken osteoclast precursors whose differentiation into mature osteoclasts is dependent upon vitamin D (Xu et al., 1993).

The effect of modulating calreticulin levels on the glandular differentiation of normal mammary epithelial cells is examined utilizing a cell culture model of differentiation (Darcy, 1991). Since steroid hormones such as estrogen and progesterone play crucial roles in this differentiation process, the effect of calreticulin on this system is determined. Similarly, the effect of modulating calreticulin expression in mammary carcinoma cell response to estrogen, Tamoxifen, progesterone and retinoic acid is determined. Since in cell lines such as MCF-7, estrogen induces proliferation, whereas Tamoxifen and all-trans retinoic acid inhibit proliferation (Pratt *et al.*, 1993), modulating calreticulin levels results in the augmention of one response preferably over another one. Modulation of calreticulin levels is therapeutically significant in the control of breast cancer.

In addition to altering calreticulin levels by cDNA transfection, we determine whether calreticulin expression is modulated by growth factors, cytokines or steroid hormones and vitamins themselves. Although the promoter of the human calreticulin gene has been cloned and characterized (Michelak, 1992), it does not give any specific clues as to its regulation. In

addition, it is conceivable that many compounds could regulate calreticulin levels at a post-transcriptional level. Factors which are known to influence the proliferation and differentiation of the above cell types (e.g. IGF-1 and vitamin D for osteoblasts; IL-6 for osteoclasts; EGF, FGF and TGF-b for mammary epithelial cells) are evaluated initially. We have already determined that 1,25 dihydroxyvitamin D3 upregulates calreticulin mRNA levels in the MC3T3 cells. Knowledge about the endogenous regulation of expression of calreticulin allow *in vivo* manipulation of nuclear hormone receptor-calreticulin interaction.

Example 9: Constitutive Expression of Calreticulin in Osteoblasts Inhibits Mineralization

The active form of vitamin D, 1a,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, also named calcitriol], is a known modulator of bone cell growth and function (reviewed in Narbaitz, 1992). Vitamin D mainly exerts its pleiotropic effects following binding to its specific receptor, which is itself a member of the steroid hormone receptor superfamily (Fuller, 1991). The ligand-bound vitamin D receptor (VDR) then interacts with its cognate binding site, termed vitamin D-response element (VDRE), to affect the transcription of target genes (see Ozono et al., 1991 for review). In osteoblastic cells, vitamin D can stimulate the activity of alkaline phosphatase (Kurihara et al., 1986), and the expression of type I collagen (Kurihara et al., 1986), osteocalcin (Yoon et al., 1988; Demay et al., 1989) and osteopontin (Noda et al., 1990). Functional VDREs have been identified and characterized for the promoters of the osteocalcin (Kerner et al., 1989; Demay et al., 1990) and osteopontin (Noda et al., 1990) genes.

We have used the osteoblastic MC3T3-E1 cell line (Sudo et al., 1983) to investigate the putative regulatory function of calreticulin in bone cells. We report that purified calreticulin inhibited the binding of VDR homodimers and VDR-RXR heterodimers to the previously characterized osteopontin and osteocalcin VDREs. Direct protein/protein interactions between the VDR and calreticulin were demonstrated. The expression of the endogenous calreticulin gene was shown to be down-regulated during osteoblastic differentiation of MC3T3-E1 cells. Constitutive expression of calreticulin

achieved by transfection of MC3T3-E1 cells with calreticulin expression vectors inhibited basal and vitamin D-induced stimulation of osteocalcin expression. However, the stimulation of the expression of osteopontin by vitamin D was unaffected. Mineralization was also inhibited in the calreticulin-expressing clones, as assessed by the reduction in calcium incorporation into the extracellular matrix and the complete absence of mineralization nodules. These results support a role for calreticulin in the regulation of osteogenesis through interaction with specific nuclear hormone receptor-mediated pathways.

Material and Methods

Gel Retardation assays

The VDR and RXRb cDNAs were gifts from Dr. J. W. Pike (Ligand Pharmaceuticals, San Diego, CA) and Dr. V. Giguère (McGill University, Montréal, QC), respectively, and were in vitro transcribed and translated using the TNT coupled wheat germ lysate system following the instructions of the manufacturer (Promega Corp., Madison, WI). Purified recombinant VDR and RXRa expressed in insect cells using baculovirus expression vectors (MacDonald et al., 1991) were provided by Dr. M. Haussler (University of Arizona, Tucson, AZ). For gel retardation assays, 5 ml from the TNT reactions or 40 ng of the baculovirus-expressed receptors were incubated with or without purified calreticulin (Rojiani et al., 1991) together with 4 x 10^3 cpm of probe in gel retention mix [10mM Tris-HCl (pH 7.3), 50mM NaCl, 1mM DTT, 1mM EDTA, 2mM $MgCl_2$, 0.2% Nonidet P-40, 12% glycerol, 1.3 mg/ml BSA, 10^{-7} M 1,25-$(OH)_2D_3$] in a final volume of 25 ml. Nonspecific competitor DNA consisted of 8 µg poly (dI-dC) and 0.2 µg salmon sperm DNA or 500 ng of poly (dI-dC) for the in vitro translated and baculovirus-expressed receptors, respectively. The receptor and calreticulin proteins were preincubated for 30 minutes on ice prior to addition of the probe DNA. The probes used were synthetic oligonucleotides corresponding to the murine osteopontin VDRE (Noda et al., 1990) or the murine osteocalcin VDRE (Rahman et al., 1993). Probes were labeled by Klenow fill-in or kinasing (St-Arnaud and Moir, 1993). After incubation for 20 minutes at room temperature, bound probe was

separated from free oligonucleotide on a 7% 30:1 acrylamide:bisacrylamide non-denaturing gel in 1x TBE (89mM Tris Borate [pH 8.3], 2mM Na_2.EDTA). Samples were migrated at 150V for 3 hours with recirculating 1x TBE. Gels were then dried and autoradiographed.

Immunoprecipitation

The VDR was labeled with ^{35}S-methionine using the in vitro express translation kit as instructed by the manufacturer (Stratagene Corp., LaJolla, CA). The labeled receptor was incubated for 60 min on ice with or without purified calreticulin (Rojiani et al., 1991) in RIPA buffer (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate) prior to addition of 10 to 30 ml of anti-calreticulin antibody (Lieu et al., 1988). To ascertain that VDR and calreticulin interacted through the KXFFK/RR motif, an additional immunoprecipitation reaction included 100 µg of the synthetic peptide KLGFFKR (Dedhar et al., 1994). Immunoprecipitation reactions were incubated for 4 days at 4°C and antigen-antibody complexes were collected by the addition of Protein A-sepharose (Pharmacia Canada, Baie d'Urfé, QC). The immunocomplexes were washed four times in RIPA buffer prior to analysis by SDS-PAGE on a 7.5% gel. The fixed, dried gel was subsequently autoradiographed.

Cells and Tissue Culture Conditions

The MC3T3-E1 cells were maintained as previously described (Candeliere et al., 1991). Mineralization was accelerated by supplementing the medium with 10% fetal bovine serum, 50 µg/ml ascorbic acid, and 5 mM b-glycerophosphate (Ecarot-Charrier et al., 1983). Cultures were stained for mineralization nodules using the von Kossa method (Mallory, 1942).

RNA Extraction and Analysis

Cells were cultured for 14 days in medium supplemented for mineralization and then treated with 10^{-8} M 1,25-$(OH)_2D_3$ in ethanol or vehicle alone for 24 h. Total RNA was isolated by the lithium chloride-urea technique of Auffray and Rougeon (1980). Northern blot hybridization was performed as described elsewhere (St-Arnaud et al., 1988). The probes used included the 1.9 kb Sac I fragment from the human calreticulin cDNA

(McCauliffe et al., 1992); a 470 bp EcoR I-to-Pst I fragment from the murine osteocalcin cDNA (Celeste et al., 1986); the full-length murine osteopontin cDNA (Smith and Denhardt, 1987); and the MAT1.1 probe for mouse alpha-tubulin (Lemishka et al., 1981).

Expression Vectors and Transfections

The SV40 promoter-driven pECE-Cal expression vector was constructed by subcloning the 1.9 kb Hind III-calreticulin cDNA fragment from Cal-2 (Dedhar et al., 1994) into the corresponding site of the pECE vector (Ellis et al., 1986) in the sense orientation. Twenty µg of pECE-Cal together with 0.3 µg of the pGEM7(KJ1)R selection plasmid (Rudnicki et al., 1992) were transfected into MC3T3-E1 cells by electroporation (2 x 10^6 cells; 300V; 1000 mF; 0.2 cm electrode gap). To establish control clones, MC3T3-E1 cells were transfected with 2 µg of pGEM7(KJ1)R selection plasmid alone. Following electroporation, the cells were plated in 100 mm-diameter dishes. Selection was initiated the next day by adding 1.2 mg/ml G-418 (Canadian Life Technologies, Burlington, ON) to the culture medium. Three control clones and four clones transfected with pECE-Cal were picked and established. The clones were maintained as the parental MC3T3-E1 cells except that the culture medium included 200 µg/ml of G-418. Expression of the recombinant calreticulin protein in transfected clones was assessed by Western blotting of whole cell extracts with an anti-calreticulin antibody (Rokeach et al., 1991) as previously described (Dedhar et al., 1994).

Assay of ^{45}Ca accumulation

Accumulation of ^{45}Ca into the matrix layer was measured as described by Matsumoto et al. (1991). Cells were cultured for 14 days prior to treatment with 10^{-8} M 1,25-$(OH)_2D_3$ or vehicle for 2 days.

Results - Calreticulin Inhibition of DNA Binding by VDR

We used gel retardation assays with in vitro-translated or baculovirus-expressed VDR and RXR proteins to determine the effect of purified calreticulin on the binding of VDR homodimers and VDR/RXR heterodimers to characterized VDREs. Figure 4A shows that calreticulin inhibited the binding

of the heterodimer to the murine osteopontin VDRE (Noda et al., 1990) in a dose-dependent manner (lanes 8-11). A similar result was obtained using the VDRE from the promoter region of the murine osteocalcin gene (Rahman et al., 1993) (Fig.4B, lanes 3-6). The amount of calreticulin required for the inhibition of VDR binding to each VDRE was not significantly different. The inhibition by calreticulin was also observed with VDR homodimers (Fig.4A, lanes 4-7), demonstrating that calreticulin can interact with the VDR protein, and that the inhibition of DNA binding was not mediated solely through interaction of calreticulin with RXR. Unprogrammed wheat germ lysate (Fig. 4A, lane 2) or purified calreticulin alone (Fig.4A, lane 3 and Fig.4B, lane 2) did not bind to the VDRE probes.

Recent results have suggested that calreticulin interacts directly with nuclear hormone receptors through a conserved KXFFK/RR amino acid motif nested within the DNA-binding domain of the receptor proteins (Dedhar et al., 1994; Burns et al., 1994). We addressed the VDR-calreticulin interaction using immunoprecipitation reactions with labeled VDR, purified calreticulin, and anti-calreticulin antibodies. Antibodies directed against calreticulin did not immunoprecipitate the in vitro-labeled VDR (Fig.4C, lane 1). However, when purified unlabeled calreticulin was added to the reaction, the labeled VDR protein was co-immunoprecipitated by the anti-calreticulin antibodies (Fig.4C, lane 2). This result demonstrated a direct interaction between VDR and calreticulin. This reaction was mediated through the KGFFRR motif of the VDR, as a synthetic KLGFFKR peptide added simultaneously with calreticulin competed for the interaction of the VDR with calreticulin and substantially decreased the amount of co-immunoprecipitated labeled receptor (Fig.4C, lane 3).

Calreticulin Expression during Osteoblastic Differentiation

MC3T3-E1 cells exhibit a pre-osteoblastic phenotype during exponential growth. Upon reaching confluency, the cells begin to express osteoblastic phenotype markers such as alkaline phosphatase and type I collagen (Sudo et al., 1983). Long-term cultures form multilayers of cells that express late osteoblastic differentiation markers and form calcified bone

tissue (nodules of mineralization) in vitro (Sudo et al., 1983). This pattern closely recapitulates the osteoblastic differentiation sequence (Stein et al., 1990; Zhou et al., 1994).

We extracted RNA from sub-confluent, confluent and mineralizing cultures of MC3T3-E1 cells to analyze the expression of the endogenous calreticulin gene. Figure 5, panel B shows that the 1.9 kb calreticulin mRNA could be detected in early and confluent cultures (lanes 1 and 2, respectively). No calreticulin expression could be detected in mineralizing cultures (Fig.5B, lane 3). Fig.5A shows that comparable amounts of total RNA were loaded in each lane. This pattern of expression of the endogenous calreticulin gene was observed in two independent experiments (data not shown).

We also probed the RNA blot for expression of osteoblastic phenotype markers. Fig. 5C shows that the osteopontin gene was expressed at all stages of culture. Thus osteopontin expression could be detected in cultures that did or did not express the endogenous calreticulin gene (compare Fig.5, panels B and C). On the contrary, the osteocalcin mRNA was only detected in mineralizing cultures (Fig. 5D). Thus osteocalcin gene expression appeared to coincide with the inhibition of endogenous calreticulin gene expression in the cultured cells (Fig.5B and 5D, lane 3).

Expression of Recombinant Calreticulin

We isolated four clones stably transfected with the pECE-Cal vector (clones SV-Cal 4, SV-Cal 5, SV-Cal 6, and SV-Cal 12). Three clones transfected with the selection vector alone served as controls.

Figure 6 shows the relative expression levels for the calreticulin protein in parental MC3T3-E1 cells and the control and transfected clones. Elevated calreticulin protein expression was achieved in all four clones transfected with the calreticulin expression vector. The increase in calreticulin expression was estimated to vary between 2- to 5-fold over parental levels. The expression of the recombinant calreticulin protein was constitutive and maintained in long-term cultures of the transfected clones (data not shown). In both parental MC3T3-E1 cells and transfected clones, calreticulin was detected in the

nucleus and the perinuclear region using immunocytochemistry (not shown), as demonstrated for other cell types (Dedhar, 1994); the relative abundance of nuclear calreticulin was increased in the clones transfected with the calreticulin expression vector (data not shown).

Enhanced calreticulin expression affected the morphology of the cells. This change was more evident in early cultures that had just reached confluency. The clones overexpressing calreticulin had a spindle-shaped appearance compared to the characteristic cuboidal osteoblastic morphology of the parental cells and the control clones (Fig.7). This morphological change became less apparent in long-term, multilayer cultures (data not shown).

Calreticulin Overexpression Selectively Inhibits Gene Expression

Long-term (14 days) cultures of control and calreticulin-expressing clones were treated with 1,25-$(OH)_2D_3$ for 24 h and total RNA was extracted to analyze the influence of calreticulin overexpression on vitamin D-stimulated gene expression in bone cells. Fig. 8 shows that constitutive calreticulin expression inhibited the induction of osteocalcin expression by vitamin D in all clones studied. Basal levels of osteocalcin mRNA were also drastically down-regulated in calreticulin expressing clones (Fig.8). These observations are consistent with the pattern of osteocalcin gene expression observed in parental cells, where osteocalcin mRNA could not be detected in subconfluent and confluent cultures of MC3T3-E1 cells that expressed endogenous calreticulin mRNA (Fig. 5). Similarly, vitamin D treatment of subconfluent and confluent MC3T3-E1 cultures did not induce osteocalcin gene expression (data not shown). Interestingly, the vitamin D-induced stimulation of the expression of the osteopontin gene was unaffected by calreticulin (Fig. 8). This result is in accord with the data obtained in parental cell cultures, where osteopontin expression could readily be detected in early cultures of MC3T3-E1 cells that expressed significant levels of endogenous calreticulin mRNA (Fig. 5). Thus our observations suggest that calreticulin overexpression had specific effects on the modulation of gene expression by nuclear hormone receptors and did not inhibit gene expression in general. Indeed, the expression of alpha-tubulin, which was analyzed to monitor for

equal loading of RNA for each sample, was unaffected by calreticulin overexpression (Fig. 8).

Constitutive Calreticulin Expression Inhibits Mineralization

Multilayer cultures of MC3T3-E1 cells deposit mineral and form calcified bone tissue after 14 to 21 days in culture (Sudo et al., 1983). This osteogenic process can be documented by counting the number of mineralization nodules and by measuring the incorporation of labeled calcium into the extracellular matrix. The calcium accumulation into the matrix has been shown to be stimulated by treatment of the cultures with 1,25-$(OH)_2D_3$ (Matsumoto et al., 1991). We have used both assays to analyze the influence of constitutive calreticulin expression on mineralization in osteoblastic cells.

Fig. 9, panel A shows a 14 day old culture of the control clone G7KJ1-2 that was stained for mineralization nodules using the von Kossa method (Mallory, 1942). Long-term cultures of all three control clones yielded similar results that were representative of the mineralization observed in parental MC3T3-E1 cultures (not shown). Fig.9B illustrates the complete absence of mineralization nodules observed in calreticulin overexpressing clones. The data from a typical mineralization experiment is summarized in Table VI5. The cultures of the control clone G7KJ1-2 contained 53 ± 4 nodules (mean ± S.E.M., n=4) whereas the cultures from all the clones expressing calreticulin constitutively contained no nodules (0.6 ± 0.3; mean ± S.E.M., n=2 for each of the four clones).

Table VI.

Number of mineralization nodules in long-term cultures of control and calreticulin-transfected clones

Clone	Number of nodules	Mean ± S.E.M.
G7KJ1-2 (control)	47; 52; 50; 63	53 ± 4
SV-Cal 4	2; 0	
SV-Cal 5	0; 0	
SV-Cal 6	0; 1	
SV-Cal 12	0; 2	0.6 ± 0.3

Cells were grown for 14 days in media supplemented for mineralization, then stained using the von Kossa method. Mineralization nodules were counted manually. The control clone G7KJ1-2 was grown in quadruplicate whereas the calreticulin-transfected clones were cultured in duplicate dishes.

Vitamin D-induced stimulation of ^{45}Ca incorporation into the extracellular matrix was also inhibited in calreticulin transfected clones. We observed the previously reported 2-fold stimulation of calcium incorporation into the matrix by vitamin D (Matsumoto et al., 1991) in cultures of MC3T3-E1 cells and in cultures of all three control clones (Fig. 10). Constitutive expression of calreticulin inhibited calcium incorporation in response to vitamin D treatment in all four clones (Fig. 10).

Discussion

We have shown that constitutive expression of calreticulin in osteoblastic cells inhibited mineralization. This inhibition was most likely mediated through specific modulation of gene expression by calreticulin, as we have demonstrated that calreticulin could interact with the vitamin D receptor to inhibit its binding to cognate response elements in vitro, and selectively inhibit certain vitamin D-mediated transcriptional responses in vivo. Our results support a role for calreticulin in the regulation of osteoblastic differentiation and function. This is significant for any disease that implicates an imbalance of bone turnover. Thus calreticulin, its mimetics or inhibitors

could be used to treat any bone disorder. Examples include a bone disorder selected from a group consisting of osteoporosis, osteopotrosis, osteopenia, rickets, osteomalacia and osteodystrophy. Osteopenia could be steroid induced osteopenia or drug induced osteopenia.

The skeleton is the body's major repository of calcium in the form of hydroxyapatite crystals. Mineralization, i.e. the deposition of mineral along the fibrils of the collagen matrix of bone, is under the control of the osteoblast (Termine, 1993). But the role of the osteoblast is not to act as a calcium-storage compartment since a proportion of the skeletal content of calcium is freely exchangeable with the extracellular fluids and serves as the major storage pool of calcium (Broadus, 1993). Moreover, the function of the osteoblast is not dependent on the availability of a large intracellular store of calcium, in contrast, for example, to the function of skeletal myotubes which require rapid calcium release to initiate muscle contraction. Thus it is likely that the role of calreticulin in osteoblasts is not limited to a calcium storage function.

Indeed, we have shown that calreticulin can influence gene expression and mineralization. The inhibition of mineral deposition cannot be simply explained by the sequestration of calcium inside the cells by the recombinant calreticulin protein. First, the levels of expression that we achieved in the transfected clones were not disproportionately high. Second, under the conditions used, the extracellular fluid was supersaturated with regard to calcium and phosphate. In experiments measuring the stimulation of labeled calcium into the extracellular matrix by vitamin D (Fig. 10), the calreticulin-expressing clones had similar baseline amounts of ^{45}Ca incorporated in the matrix (not shown). The mechanisms responsible for the actual inhibition of calcium incorporation when the calreticulin-transfected clones were treated with vitamin D are unclear. Vitamin D treatment had no effect on the expression of the endogenous calreticulin gene in MC3T3-E1 cells (J.P. and R.St-A., unpublished observations).

In addition to vitamin D, a number of steroid hormones or morphogens binding to members of the nuclear hormone receptor superfamily can

modulate gene expression in osteoblasts. In MC3T3-E1 cells, glucocorticoids have been shown to inhibit prostaglandin E_2 synthesis (Hughes-Fulford et al., 1992) and increase the expression of betaglycan (Nakayama et al., 1994). Estrogen receptors are expressed in osteoblasts (Eriksen et al., 1988) and 17-b estradiol down regulates the transcriptional activity of the interleukin-6 gene promoter and inhibits the expression of the glycoprotein 130, a subunit of cytokine receptors [Manolagas, S.C. 1995. Bone. 16 (Suppl. 1):91S.]. Retinoic acid stimulates the expression of alkaline phosphatase and induces differentiation of preosteoblastic cell lines (Ng et al., 1988; Heath et al., 1989). All of these receptor-mediated responses represent putative targets for modulation by calreticulin. Our results, showing that calreticulin inhibited some but not all of the transcriptional responses mediated by the vitamin D receptor, suggest that certain of these nuclear hormone receptor-dependent pathways will be left intact while others may be affected. It would be interesting to investigate transcriptional responses to various steroids in bone cells expressing calreticulin constitutively.

The response to vitamin D was not completely abrogated in clones expressing the recombinant calreticulin protein (Fig. 8). This could suggest that the VDR was in stochiometric excess over calreticulin in the transfected clones, thus allowing the excess of ligand-bound VDR to escape inhibition by calreticulin and activate transcription. Indeed, vitamin D treatment has been shown to induce the expression of VDR mRNA and protein in bone cells (Mahonen et al., 1990). Alternatively, the residual vitamin D response observed in calreticulin-transfected clones could be due to the documented non-genomic effects of vitamin D (reviewed in Norman et al., 1992), which may not be affected by constitutive calreticulin expression.

Our results have demonstrated that calreticulin inhibits VDR binding to both the osteopontin and the osteocalcin VDRE (Fig. 7). However, the two genes responded dissimilarly when the calreticulin-expressing clones were challenged with vitamin D (Fig. 8). Despite differences between the sequences of the two VDREs (Kerner et al., 1989; Demay et al., 1990; Rahman et al., 1993) that could affect VDR binding affinity to each response

element (Nishikawa et al., 1993), we were unable to detect any difference in the capacity of calreticulin to inhibit VDR binding to each VDRE. Thus the structure of the response element cannot seem to account for the variation in transcription. It is more likely that the expression of each gene is dictated by the structure of the entire promoter region, and not just a single element. The pattern observed in the parental cells supports this view. Indeed, the osteopontin mRNA was readily detected in early cultures of MC3T3-E1 cells that also expressed calreticulin (Fig. 5). On the contrary, osteocalcin transcripts were only observed in long-term cultures that had down-regulated calreticulin expression (Fig. 5). Thus it appears that calreticulin expression inhibits both steady-state and stimulated osteocalcin expression, whereas it does not affect the transcription of the osteopontin gene. In this respect, the pattern of expression of these two osteoblastic phenotype markers that we measured in the calreticulin-expressing clones closely mimicked what was observed in the parental MC3T3-E1 cells.

There was no correlation between the level of calreticulin overexpression achieved in independent clones and the inhibition of basal and stimulated osteocalcin transcription, as all clones exhibited an identical blunted osteocalcin response (see Fig. 6 and Fig. 8). Our results suggest that it was not the magnitude of the expression of the recombinant protein per se that affected the osteocalcin response, but the fact that calreticulin expression was constitutive and therefore not down-regulated in long-term cultures of the transfected clones. Again, this observation parallels the pattern of osteocalcin gene expression that we documented in non-transfected MC3T3-E1 cultures (Fig. 5).

Attachment to extracellular matrix components modulates gene expression and differentiation of bone cells. For example, differentiation of canalicular cell processes was observed following contact of MC3T3-E1 cells with laminin (Vukicevic et al., 1990), and gene expression is affected when preosteoblastic cells are plated on various extracellular matrix components (Traianedes et al., 1993). Osteoblasts express various forms of the integrin family of cell surface receptors for matrix proteins (Hughes et al., 1993; Saito

et al., 1994), and inhibition of integrin function using subunit-specific antibodies has been shown to prevent cytokine-induced osteoblastic differentiation of osteosarcoma cells (Dedhar, 1989). Calreticulin binds to a-integrin subunits (Leung-Hagesteijn et al., 1994) and recent results suggest that this interaction can modulate the affinity state of integrins (Dedhar, 1994). Moreover, calreticulin can modulate nuclear hormone receptor-dependent gene expression (Dedhar et al., 1994; Burns et al., 1994). Taken together, these observations support the existence of a calreticulin-modulated signal transduction pathway linking substratum attachment via integrin receptors to the control of gene expression. Our results further support an important role for this pathway in the regulation of osteoblastic differentiation and function.

Example 10 Modulation of Hormone Receptor-Calreticulin Interaction by Peptides, Peptide Mimetics and Antibodies

We have demonstrated that synthetic peptides based on the sequence KXFFYR can behave as competitive inhibitors of calreticulin-nuclear hormone receptor interaction (see Example 7). This was demonstrated by gel mobility shift assays. When incubated with the nuclear hormone receptor and calreticulin, the peptides can reverse the ability of calreticulin to inhibit receptor-DNA binding *in vitro*. Since a scrambled peptide was completely ineffective, this assay can distinguish peptide specificity. These data suggest that the interaction of calreticulin with the nuclear hormone receptors can be manipulated with such peptides.

We use the gel mobility shift assay and androgen receptor as well as the retinoic acid receptors (Shago *et al.*, 1994) to determine which amino acids within the KXFFYR sequence are critical for the calreticulin-receptor interaction. This is done by synthesizing peptides with single amino acid substitutions and then testing them for their activity in gel mobility shift assays as described by us previously (see Example 7). Results from these experiments identify the critical amino acids in this sequence motif required for calreticulin-receptor interaction.

The nuclear hormone receptors can be subdivided into two categories, the steroid receptors, which include the androgen receptor, glucocorticoid

receptor, mineralocorticoid receptor and estrogen receptor; and the thyroid hormone/retinoic acid receptor group which includes the retinoic acid receptors, thyroid hormone receptor and vitamin D receptor. Unlike the first category, this latter category of receptors bind to their DNA response elements as heterodimers with RXR. The above experiments therefore define the sequence motif for a receptor from each of these two categories i.e. androgen receptor and retinoic acid receptors (RAR/RXR; RXR/RXR). Subsequent experiments are then be carried out with other receptors such as estrogen receptor and vitamin D receptor.

The N-domain of calreticulin (Michalak et al, 1992) has been implicated in the interaction with the glucocorticoid receptor. We have prepared GST-fusion proteins in *E. coli* consisting of either full length human calreticulin, the N-domain, P-domain or the C-domain. Each of these recombinant proteins is tested in gel mobility shift assays (according to the teaching of Example 3) for their effectiveness in inhibiting receptor-DNA interaction. The receptors we use initially will be the androgen receptor and the retinoic acid receptors. P19 EC cells or Vero cells are also transiently transfected with expression vectors containing the N, P or C calreticulin domains, and their effect on hormone induced gene expression determined as described by us previously (see Example 5).

We have identified the calreticulin domain which interacts with the receptors and have used proteolytic fragments of the recombinant proteins (generated by proteolytic cleavage and purification of peptides by HPLC) to further define the minimal peptide(s) necessary for mediating the interaction. If a sufficiently small peptide is found to be active, then synthetic peptides from within that sequence will be evaluated.

Example 11 Preparation and testing of delivery systems for peptide-antagonists of calreticulin nuclear hormone receptor interactions

After we identify peptides capable of inhibiting calreticulin-receptor interactions *in vitro*, we test the efficiency of these peptides on cells. To do this, the peptides are incorporated into cationic lipid vesicles (liposomes, such as lipofectin) and incubated with the cell types described in Example 10. To assess internalization of the peptides, some peptides are conjugated with fluorescein isothiocyanate (FITC). After incubation of the liposomes with the cells for different time periods, the cells are examined by immunoflourescence microscopy to assess intracellular accumulation. The biological effects of the peptides are determined by assessing hormonal sensitivity of the target cells, expression of primary response genes by Northern and Western blot analysis, and hormone-induced expression of luciferase reporter constructs containing various response elements, described in Example 6. The target cells and the hormone responsive parameters to be used are described in Example 7.

These experiments determine whether the peptides defined in Example 7 are functional at the cellular level in antagonizing hormone receptor-calreticulin interactions. Once we optimize the peptide delivery systems and achieve the predictable cellular responses, we test these peptide-liposomes in animal model systems such as bone formation in the mouse, and rat mammary gland differentiation. For the former, primary osteoblasts derived from mouse calvariae are njected into the gluteal muscle of recipient mice where these osteoblasts differentiate to form mineralized nodules. The effect of local or systemic administration of peptide-liposomes are then assessed in this model. Similarly the effect of the peptides on normal mammary gland development after injection of normal rat mammary epithelial cells into mammary fat pads (Darcy, 1991) are assessed. If the peptides are found to be effective in influencing these processes then their effect on animal models of osteoporosis, and growth and differentiation of human breast and prostate cancer xenografts in nude mice are determined.

The following examples relate to the manufacture and use of pharmaceuticals to treat particular diseases, including cancer, osteoporosis, and chronic inflammatory disease, using pharmaceuticals comprising a protein for use in modulating hormone responsiveness and a carrier.

Example 12 Method of Treating Prostate Cancer

Prostate cancer is the most frequently diagnosed invasive cancer and the second most common cause of cancer death in men in Western societies (Boring, 1993; Coffey, 1993). At present, prostate cancer patients are diagnosed with either locally invasive, or disseminated disease, and the currently available forms of treatment may prolong survival but are essentially only palliative (Scardino, 1992; Koxlowski, 1991; Santer, 1992).

Although primary endocrine ablation leads to an initial response in about 70% of patients with advanced disease, most patients relapse within three years and only about 20% survive for five years (Kozlowski, 1991). This rapid progression of prostate cancer following failure of primary hormone therapy is attributed to androgen-independent tumor growth.

In some androgen-insensitive rat as well as human prostate cancer cell lines, androgen independence is associated with a loss or decrease in androgen receptor (AR) mRNA and protein levels (Quarmby, 1990; Tilley, 1990). However some prostate carcinoma cell lines derived from metastases retain AR expression and androgen sensitivity (e.g. Ln CAP cell line). Furthermore there is evidence that some prostate cancer cells which continue to grow after initiation of anti-androgen therapy retain expression of AR (van der Kwast, 1991; Tilley, 1994). Similarly, AR expression is retained by androgen-independent mouse mammary tumors (Dabre, 1987).

These observations suggest that mechanisms other than the loss of AR expression are involved in the progression to an androgen-independent state. One explanation could be the presence of mutations in the AR gene in a subpopulation of tumor cells which results in aberrant regulation of growth by steroids. Indeed mutations in the AR gene have been detected in prostate cancer cells, although their significance in tumor progression is not yet clear.

Another explanation might be alterations in the expression or function of components which regulate AR activity and AR dependent gene expression.

As described in previous Examples, calreticulin, can bind to nuclear hormone receptors by interacting with the KXFFYR sequence. The interaction results in a profound inhibition of nuclear hormone receptor DNA binding activity which can be reversed by soluble competing synthetic peptides with the generic sequence KXFFYR.

The level of expression of calreticulin in prostate androgen-dependent and independent prostate cancer cells could have significant effects on androgen receptor activity. Furthermore, experimental manipulation of calreticulin levels in prostate cancer cells has resulted in the modulation of androgen-receptor activity.

For example, Table VII shows that the expression of calreticulin increases with protate cancer progression from benign to BPH (benign prostatic hyperplasia) to PIN (Prostatic Intraepithial Neoplasia) to Cancer. This is an important finding linking calreticulin as a marker of prostate cancer progression and also as a target for therapy as described in this application.

In addition, the interaction of androgen receptors with calreticulin could be taken advantage of, theoretically, by utilizing calreticulin or calreticulin-derived peptides and peptide-mimetics for the inhibition of androgen receptor dependent prostate cancer cell growth. Such a therapeutic strategy might be particularly useful in recurrent, androgen-independent prostate cancers which retain expression of AR or mutant ARs and which may bind to DNA in the absence of androgen.

Calreticulin expression in the nuclear, cytoplasmic and microsomal fractions from human prostate carcinoma cell-lines PC-3, DU-145, LnCAP, as well as highly invasive variants of PC-3 cells (IPC31-3) (Dedjar. 1994) is determined by Western blot analysis utilizing two different polyclonal anti-calreticulin antibodies as described by us previously (Leung-Hagesteijn, 1994).

Table VII

Patient	Normal	Atrophy	BPH	PIN	CA
Not treated					
1	1+	+/-	2+	4+	3-4+
2	1-2+	N/A	3+	3-4+	3-4+
3	1-2+	+/1	2+	4+	3-4+
4	2+	1+	2+	4+	2-3+
5	1-2+	1+	1-2+	3-4+	3-4+
Treated					
6	1+	1+	N/A	3+	2-3+
7	1-2+	1+	1-2+	3-4+	5-4+
8	1-2+	1+	1-2+	4+	3-4+
9	+/-	0	N/A	2+	1-2+
10	2+	2+	N/A	2+	2-4+

BPH: Benign prostatic hyperplasia

PIN: Prostatic intrapithelial neoplasia

CA: Carcinoma

Parallel paraffin sections from prostate biopsies were stained with Hemotoscylin and Eosin or by in situ hybridization with a calreticulin-specific antisense DNA probe. Calreticulin mRNA expression was confined to the epithelial tissue and dramatically increased, within the same section, from benign regions to PIN and CA regions. The extent of signal was evaluated independently by three pathologists. Some sections were also stained with the sense probe as control which was negative.

Expression at the level of mRNA is carried out using a 1.9 Kb calreticulin cDNA. Calreticulin expression in these cells is determined after treatment with androgens (for LnCAP cells which express AR, or for PC-3 cells transfected with AR, see below), retinoic acid, 1,25 dihydroxy vitamin D3, and growth factors such as epidermal growth factor and insulin-like growth factors.

A subset of a large (>125) tissue bank of frozen human prostate cancers (Sunnybrook Health Science Centre were treated with neo adjuvant androgen ablative therapy prior to resection. Each frozen block has been histologically characterized. The bank also contains hormone-resistant prostate cancer specimens and will accrue fresh bond marrow metastases from warm autopsies on patients dying of androgen resistant prostate cancer. These tissues are freely available for the aforementioned analyses. This permits determination of calreticulin expression in untreated, hormonally treated and hormone resistant disease.

Calreticulin expression in cryostat sections is determined by immunohistochemistry using the avidin-biotin complex method described by Hsu *et al.*, 1981 as well as by *in situ* hybridization using antisense cDNA calreticulin probes as described by Naylor *et al.*, 1990. These procedures are performed in Dr. Malik's laboratory where they are carried out on a routine basis. Simultaneous determination of the androgen-receptor status in these tissues on serial sections are carried out using anti-AR antibodies (Tilley, 1994).

Overexpression, or inhibition of expression of calreticulin is carried out by stable transfection of sense (pRC-CMV-Cal1) or anti-sense (pRC-CMV-Cal2) (Dedhar, 1994) cDNA expression vectors into LnCAP or AR expressing PC-3 cells. AR expressing PC-3 cells are obtained from Dr. Paul Rennie, Vancouver, B.C. We have previously described the utility of these calreticulin expression vectors in manipulating hormone responsiveness (see Example 4). These cells are also transfected with a tetracyclin inducible calreticulin expression vector. This expression plasmid, pUHD10-3-CAL, has been constructed and calreticulin sense or antisense mDNA expression is induced via a tetracyclin-operators. Calreticulin expression levels are determined by Western blot analysis as described above. The responsiveness of the transfected cells to androgens, in terms of cell growth, is then determined. Cell growth is determined by counting cell numbers as well as by ^{3}H-thymidine incorporation. Calreticulin overexpression makes the cells non-responsive to androgens, whereas inhibition of calreticulin expression makes the cells more sensitive, as was the case for retinoic acid responsiveness in ECP19 cells (see

Example 5). Stable overexpression of calreticulin does not alter intracellular calcium concentrations, and therefore the observed effects on hormone sensitivity are not due to effects on calcium levels.

The calreticulin transfected cells is compared with the parental or mock transfected cells for their abilities to form tumors in nude mice upon subcutaneous inoculation, or orthotopic inoculation into the prostate gland.

The domain of calreticulin which interacts with the KXFFYR sequence has been identified as the globular N-domain. This domain contains a putative ATP binding site and recombinant calreticulin can be phosphorylated *in vitro* on a serine residue within this domain (Leung-Hagesteijn, 1994). We have prepared GST-fusion proteins in *E.Coli* consisting of the full length human calreticulin, the N-domain, P-domain, or the acidic C-domain. Each of these recombinant peptides is tested in gel mobility shift assays for their effectiveness in the inhibition of *androgen receptor-DNA interaction* (see Example 10). These domains are tested for their effectiveness in inhibiting androgen mediated gene expressing by transiently expressing them in LnCAP or AR-expression PC-3 cells (described previously). Once we have identified the calreticulin domain which interacts with the androgen receptor (although this is likely to be the N-domain, based on previous work, see above), we derive proteolytic fragments from these proteins (by limited proteolytic cleavage using various proteases, and purification of peptides by high pressure liquid chromatography), and utilize these in gel mobility shift assays to further define the minimal peptide sequence required for interaction with the androgen receptor. If a sufficiently small peptide is found to be active, then synthetic peptides from within that sequence are evaluated further.

Peptides capable of inhibiting AR-DNA interactions *in vitro* are identified. In order to test the efficiency of these peptides in cells, the peptides are incorporated into cationic lipid vesicles (liposomes, such as lipofectin) and incubated with the cell types (see Example 11).

These experiments determine whether the peptides defined above are functional at the cellular level in antagonizing hormone receptor-DNA interactions. Once we have optimized the peptide delivery systems and

achieve the predictable cellular responses, we test these peptide-liposomes in animal model systems described above.

These peptides, in conjunction with current protocols of androgen ablation, are useful in inhibiting androgen-receptor mediated prostate cancer cell growth. This strategy is useful not only in the early treatment of androgen-sensitive tumors, but also in more advanced androgen-resistant tumors which may express normal or mutated ARs which can induce cell growth in an androgen-independent manner. In such tumors, the maintenance of high levels of calreticulin expression, or of adminstration of calreticulin-based peptides (derived as described above), or peptide mimetics, provide a new mode of therapeutic intervention in the inhibition of prostate cancer cell growth and progression.

Example 13 Calreticulin Modulates Osteogenesis in Vivo

The Examples in this patent application have shown that calreticulin is expressed in bone cells and that constitutive, enforced expression of calreticulin in osteoblasts perturbs steroid hormone receptor-dependent gene expression and inhibits mineralization (see below). Thus, calreticulin expression is tightly regulated during osteogenesis, and perturbations in calreticulin expression or function in bone cells are involved in the molecular etiology of specific bone diseases.

We propose to determine the pattern of expression of calreticulin during osteogenesis in vivo; compare the effects of constitutive expression of calreticulin and calreticulin mutants on mineralization by osteoblastic cells; use synthetic peptides based on the calreticulin binding motif to steroid hormone receptors to modulate the calreticulin/receptor interaction and the response of osteoblastic cells to steroids; overexpress calreticulin in osteoblasts in transgenic mice to study the role of calreticulin on bone formation in vivo; and inactivate the calreticulin gene in mice via homologous recombination in embryonic stem cells.

The work broadens our understanding of normal and impaired bone development by providing new insight into the expression and function of calreticulin in osteoblasts. The experiments show a novel means of

modulating osteoblast function and bone formation. Finally, the results of the studies provide animal models of diseases affecting bone structure and function. This leads to novel therapeutic approaches for treating particular bone diseases.

Expression of calreticulin is down-regulated at the onset of mineralization in an in vitro model of osteogenesis. Moreover, enforced expression of calreticulin in differentiating osteoblastic cells was shown to inhibit mineralization in culture. To determine if calreticulin can regulate bone formation in vivo, we use gain-of-function strategies in transgenic mice. An exogenous calreticulin sequence is placed under the control of a heterologous promoter expressed in osteoblasts; this 'mini-gene' is injected into fertilized mouse eggs and transgenic mouse lines established. Osteoblast function and bone formation is analyzed in these transgenic animals using the array of techniques at our disposal.

We express the exogenous calreticulin sequence from the onset of osteoblastic differentiation and maintain its expression in terminally differentiated osteoblasts, even osteocytes. Moreover, the expression of the calreticulin transgene occurs concomitantly with the onset of ossification during development. We use the promoter region of the alpha 2 chain of collagen type I [a_2(I)] (40) to drive the expression of the exogenous calreticulin. Using an a_2(I) promoter fragment (-2000 to +54 relative to the transcription start site) linked to a lacZ reporter gene in transgenic mice, D'Souza et al. have observed strong expression of the transgene in preosteoblasts as well as differentiated osteoblasts at both intramembranous and endochondral sites of ossification. Expression of the transgene was detected as early as 14 days p.c. in osteoblasts bordering spicules of osteoid in a mineralizing cartilaginous template of bone (D'Souza et al.). The promoter was also shown to direct expression of the reporter gene in osteocytes. Thus the activity of this promoter fragment matches both the temporal and spatial pattern of expression that we wish to impose on the calreticulin transgene; moreover, it has been tested in the context of

transgenic animals. The a_2(I) promoter also drives expression of transgenes in tendons and skin; negligible activity was reported for other tissues.

We have obtained the -2300 to +54 a_2(I) promoter fragment from Dr. Benoît de Crombrugghe (M.D. Anderson Cancer Center, Houston, TX). The 2054 Hind III promoter fragment (Schmidt et al.) is blunt-ended and subcloned upstream of the murine calreticulin gene at the unique Hpa site upstream of the initiation of translation site. Although the preferred strategy involves the use of the genomic sequence to allow for higher expression of the transgene, sequences that control the down-regulation of calreticulin expression in osteoblasts (21) are contained in introns; therefore, a second transgene vector is constructed using the calreticulin cDNA. In order to easily identify the transgene in relation to the endogenous calreticulin protein, an epitope tag from the human c-myc oncoprotein is cloned in frame at the unique Sfi I restriction site within the first exon of the calreticulin transgene. Epitope tagging with human c-myc sequences has been successfully used to monitor expression of exogenous gene constructs (Lin et al.); specific monoclonal antibodies that recognize the epitope tag are commercially available. The transgene vectors are tested for functional expression by transient transfection assays in fibroblastic cells.

The transgenic vector DNA is digested free of bacterial plasmid sequences and purified using the Gelase protocol of Dickinson et al. The purified DNA is microinjected into fertilized eggs and transgenic founder animals is derived following standard techniques of reimplantation into foster mothers.

Transgenic founder animals and progeny are identified by Southern blot analysis of tail DNA using an oligonucleotide probe corresponding to the human c-myc epitope tag. The development of the transgenic animals is then followed with particular emphasis on bone formation and osteoblast function. The expression of the transgene is initially documented in bone tissue using immunohistochemistry with the myc tag antibody (Lin et al.). Bone structure is then studied, first using whole skeletal staining with alcian blue and alizarin red, then by careful histomorphometric analysis of bone sections (Glorieux et

al.). Expression of osteoblast phenotype markers in transgenic bones is analysed by Northern blot assay and in situ hybridization.

We then establish osteoblastic cell lines from the calvaria of transgenic animals (Escarot-Charrier et al.; Sudo et al.). If clonal cell populations are derived, they are extensively characterized with regard to gene expression patterns, hormonal response, and capacity to mineralize in vitro. Treatment with the synthetic peptides based on the KXFFK/RR motif is used to attempt to block the function of the calreticulin transgene and modulate the differentiation and function of the cells.

These studies confirm the role of calreticulin in the regulation of bone formation in vivo. The gain-of-function mutation achieved in the transgenic animals is complemented with studies using a loss-of-function approach.

The advent of gene targeting technology, sometimes referred to as "gene knock-out", has allowed considerable insight into the role and function of particular gene products during development and differentiation. Briefly, the technique relies on the use of pluripotent embryo-derived stem (ES) cells (Capecchi, M.R.). An inactivating mutation is engineered into a cloned genomic fragment of the target gene and this mutated gene is introduced into ES cells cultured in vitro. Although the transfected mutant gene most frequently integrates randomly into the host cell's genome, powerful selection schemes have been designed that allow the identification and isolation of the rare cells that have incorporated the mutant gene at the corresponding targeted chromosomal location through homologous recombination (Mansour et al.; Capecchi, M.R.), thus creating a null allele of the target gene. These cells are then micro-injected into the blastocoel cavity of a preimplantation mouse embryo and the blastocyst is re-implanted into the uterus of a foster mother. Strains of mice with different coat colors are normally selected for the ES cell population and the recipient blastocyst, thus allowing simple identification of the chimeric animals on the basis of fur color. Back-crossing breeding then allows to determine if the ES cells have contributed to the germ line of the chimeric animals. The progeny that shows ES cells germ line transmission is genotyped to detect the animals that carry the engineered

mutation. These heterozygote siblings are then interbred to obtain animals that are homozygous for the desired mutation.

The ES cell technology is currently functional in our laboratory. We have successfully targeted the 25-hydroxyvitamin D 24-hydroxylase (24-OHase) gene (St. Arnaud et al.). Targeted ES cells were injected into mouse blastocysts by an outside facility, the MRC Centre of Excellence for Transgenesis, as is also planned for engineering the proposed calreticulin 'knock-out' strain. One of the resulting chimeric mice has transmitted the targeted allele to its progeny; animals that are heterozygous for the engineered mutation are normal and fertile. Animals homozygous for the targeted 24-OHase mutation are born with the expected Mendelian frequency of 25% (36/154); however, about one-half of the homozygotes died within one week after birth. We suspect that the incomplete penetrance of the homozygous phenotype may be due to the mixed genetic background (129Sv x C57 Bl 6) of the animals and are currently back-crossing the mutation into the inbred 129Sv background. Preliminary data suggest a mild hypercalcemia in homozygous mutants; histological examination revealed normal bone structure. Homozygous animals that survive are fertile. Interestingly, preliminary results suggest that bone development was abnormal in homozygotes born of homozygous females. These analyses should yield valuable insight into the biological role of $24,25(OH)_2D_3$ in mineral homeostasis and bone development. In the context of this application, they document our ability to engineer 'knock-out' strains of mice.

We have already isolated ES cell clones in which one allele of the calreticulin gene has been targeted. These clones are expanded and then injected into C57BL/6 embryos at the blastocyst stage using standard techniques. This last step is performed on a cost basis by the MRC Centre of Excellence for Transgenesis at the Montreal General Hospital, as previously mentioned (St. Arnaud et al.).

Chimeric animals are identified on the basis of chimeric coat color (agouti patches on a black background). Chimeric males are bred to C57BL/6 females and germ line transmission assessed by the presence of the agouti

coat color in the resulting F1 progeny. Animals showing germ line transmission are genotyped by Southern blot analysis of tail DNA and heterozygotes for the mutated allele are mated *inter se* to produce all three possible genotypes (+/+, +/- and -/-). The phenotype of the +/- heterozygous and -/- homozygous animals will then be studied in detail.

Calreticulin has been shown to regulate multiple functions in various cell types: calcium binding (Michalak et al.); cell attachment via its interaction with integrin receptors; and modulation of DNA binding by nuclear hormone receptor (Dedhar et al.; St. Arnaud et al.; Burns et al.). Some of these roles of calreticulin may depend on strict stochiometric relationships between calreticulin and its target proteins. Inactivating one calreticulin allele alters this stoichiometry through a gene dosage effect and affect development of heterozygous mice. Since calreticulin has been shown to modulate the action of the AR (Dedhar et al.), GR (Burns et al.), RAR (Dedhar et al.), and VDR (St. Arnaud et al.), we concentrate on the morphology and function of some of the target tissues of these hormones: adrenal glands, gonads, lungs, and bone. Moreover, as cell attachment via the integrin receptors is compromised in heterozygous animals, they exhibit a higher incidence of carcinogenesis.

To analyze -/- homozygous mutants, animals from the F2 litters are genotyped to determine the ratio of transmission of the homozygote -/- genotype. A ratio significantly lower than the Mendelian expectation of 25% suggests an embryonic lethal mutation. We then analyze pregnant females for the presence of dead embryos at various stages of pregnancy to confirm; the embryos are genotyped by PCR to confirm the homozygosity for the null mutation. The cause of the embryonic lethality is then investigated. Considering the multiple functions of calreticulin (29), embryonic lethality represents a possible outcome of the targeted inactivation of both calreticulin alleles.

However, if live F2 mutant homozygotes are recovered, their phenotype is extensively studied. First, the expression of the calreticulin mRNA and protein is examined using Northern and Western blot assays to ascertain that the engineered mutation created a true null allele.

Various phenotypes for the -/- homozygotes is hypothesized: the mutation could have no detectable effects; this would suggest redundancy of function between calreticulin and other calcium-binding protein. The multiple functions described for the calreticulin protein (Dedhar et al.) suggest, however, that the absence of phenotype in homozygous mutants is highly unlikely.

The most interesting possibility is that homozygous animals develop a detectable phenotype: this could be related to skeletal development or not. If the latter is the case, we determine which tissue(s) is affected and what effect the lack of calreticulin function has on that tissue. Again, target tissues of steroid hormones, such as the adrenal glands and gonads, are given particular attention.

However, our results obtained using in vitro models of osteogenesis strongly suggest that bone development is impaired in calreticulin mutant animals. This is manifested by impaired growth, deformities, or abnormal histology of bone and/or growth plate cartilage. The morphology of the bones and growth plate cartilage from homozygous animals is analyzed using the resources of the Shared Bone Morphology facility. We then characterize the observed phenotype in more detail by analyzing the expression of known markers of osteoblastic differentiation (alkaline phosphatase, osteocalcin, osteopontin, etc.) using in situ hybridization to attempt to determine the effect of the absence of calreticulin function on gene expression during osteoblastic differentiation in vivo. Finally, we establish links between the phenotype of homozygous animals and diseases affecting bone and/or cartilage in humans. Our mutant strain is then used as an animal model for diseases affecting skeletal development and provide the basis for improved clinical treatment of these pathologies.

The gain-of-function and loss-of-function mutations of calreticulin that we affects osteoblast function and bone formation. The observed phenotypes are comparable to some human bone pathologies. We then analyze calreticulin expression in sample tissues from those pathologies in order to gain further insight into the molecular etiology of metabolic bone diseases.

We specifically inactivate the function of calreticulin exclusively in bone, in order to reduce the possibility of embryonic lethality upon calreticulin inactivation. This is accomplished with the novel methodology of conditional gene knock-out, which relies on the use of the site-specific Cre recombinase in a transgenic approach. These experiments allow us to develop animal models of bone diseases which prove invaluable for the elaboration of rational and effective therapeutic approaches for the pathologies affecting the bone cell.

Example 14 Method of Treating Breast Cancer

Here. we use a similar protocol as for prostate cancer (Example 12) but use proteins/mimetic selective for estrogen. Transfections are made into MCF-7, T47-D human breast cancer cells lines instead of LnCAP. For patient treatment, the peptides or their mimetics, or calreticulin or its mimetics are delivered in lipid vesicles prepared as described in Examples 11 and 12.

Example 15 Method of Treating Chronic Inflammatory Disease

The debilitating symptoms of chronic inflammatory diseases such as arthritis arise from inadvertent immune responses. Steroidal compounds are major immunosuppressive agents often used in the therapy of chronic inflammatory diseases. The response to such therapy may be dramatically augmented by the co-administration of calreticulin-hormone receptor antagonists based on the KXFFYR sequence. Such peptides, or their organic mimetics, may dramatically enhance the hormonal response by activating those receptors which may be bound by calreticulin. This may also result in the use of lower concentrations of the steroidal compounds, resulting in fewer side effects. The mode of delivery of such peptides or organic mimetics would be in lipid vesicles described in Examples 11 and 12.

Example 16 Method of Treating Osteoporosis

Osteoporosis results from an imbalance in the rate of bone resorption versus bone formation. Specifically, in post menopausal women, the decrease in systemic estrogen levels results in decreased bone formation in the face of continual osteoclast mediated bone resorption. Estrogen therapy,

by using estrogen analogs which may specifically enhance osteoblast function and bone formation is under intensive study.

Given our findings that increased expression of calreticulin inhibits bone formation, the expression of calreticulin is likely increased in patients with osteoporosis. Therefore co-administration of KXFFYR based peptides or mimetics specific for the antagonism of calreticulin-estrogen receptor interaction would be highly beneficial for this treatment. Such peptides or mimetics would dramatically increase the efficacy of estrogen analogs used in such therapy.

An alternative approach for the use of calreticulin based therapy would be inhibition of osteoclast differentiation. The differentiation of mature osteoclasts from osteoclast precursors is enhanced by Vitamin D3. The specific inhibition of the vitamin D receptor by calreticulin based mimetics would therefore result in the suppression of osteoclast mediated bone resorption. Combined therapy of increasing bone formation and down regulating bone resorption may be an effective treatment for osteoporosis.

The peptides or mimetics would once again be delivered by methods described in Examples 11 and 12.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

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The documents listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques and/or compositions employed herein.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMS ARE DEFINED AS FOLLOWS:

1. An isolated and purified product for use in modulating hormone responsiveness.

2. The product of claim 1, wherein the product inhibits hormone receptor induced gene transcription.

3. The product of claim 1, wherein the product promotes hormone receptor induced gene transcription.

4. The product of claim 2, wherein the product binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R and wherein the product is selected from a group consisting of calreticulin and a mimetic of calreticulin.

5. The product of claim 3, wherein the product comprises the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R, and wherein the product is a synthetic peptide which binds to calreticulin.

6. A product which binds to calreticulin and which reverses selectively calreticulin inhibitions of a receptor binding to DNA response elements.

7. The product of claim 6 which is a synthetic peptide.

8. The product of claim 7, wherein the receptor is retinoic acid and the product is KLDFFKR.

9. The product of claim 7 wherein the receptor is the androgen receptor and the product is selected from a group consisting of KLGFFGR and KLGFFKG.

10. An isolated DNA molecule encoding an amino acid sequence for use in modulating hormone responsiveness.

11. The isolated DNA molecule of claim 10, wherein the molecule encodes a first amino acid sequence that binds to a second amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R, and wherein

the first amino acid sequence is the sequence for calreticulin or for part of a mimetic of calreticulin.

12. A method of treating a disease in a mammal comprising regulating hormone receptor induced gene transcription in a cell.

13. The method of claim 12, further comprising regulating the activity, quantity or stability of a protein for use in hormone receptor induced gene transcription.

14. The method of claim 12, further comprising administering to the mammal a pharmaceutical comprising a protein and a carrier.

15. The method of claim 14, wherein the protein binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R.

16. The method of claim 14, wherein the protein binds to calreticulin.

17. The method of claim 14, wherein the protein is one selected from a group consisting of calreticulin and a mimetic of calreticulin, and wherein the carrier is a lipid vesicle.

18. The method of claim 12, wherein the disease is one selected from a group consisting of breast cancer, prostate cancer, promyelocytic leukemia, solid tumors, chronic inflammatory disease and arthritis.

19. The method of claim 12, wherein the disease is a bone disorder.

20. The method of claim 19, wherein the disease is one selected from a group consisting of osteoporosis, osteopotrosis, osteopenia, rickets, osteomalacia and osteodystrophy.

21. The method of claim 12, further comprising decreasing or eliminating the quantity of calreticulin present in the cell.

22. The method of claim 12, further comprising decreasing the stability of calreticulin present in a cell.

23. The method of claim 12, wherein the hormone receptor is one selected from a group consisting of: glucocorticoid receptor, mineralcorticoid

receptor, androgen receptor, progesterone receptor, estrogen receptor, retinoic acid receptor, thyroid hormone receptor, vitamin D receptor and orphan receptors.

24. A kit containing a pharmaceutical comprising a protein for use in modulating hormone responsiveness together with a carrier.

25. The kit of claim 24, wherein the protein binds to the amino acid sequence KXFFYR, wherein X is G, A or V and wherein Y is K or R and wherein the protein is calreticulin or a mimetic of calreticulin.

26. The kit of claim 24, wherein the protein binds to calreticulin.

27. The kit of claim 24, wherein the carrier is a lipid vesicle.

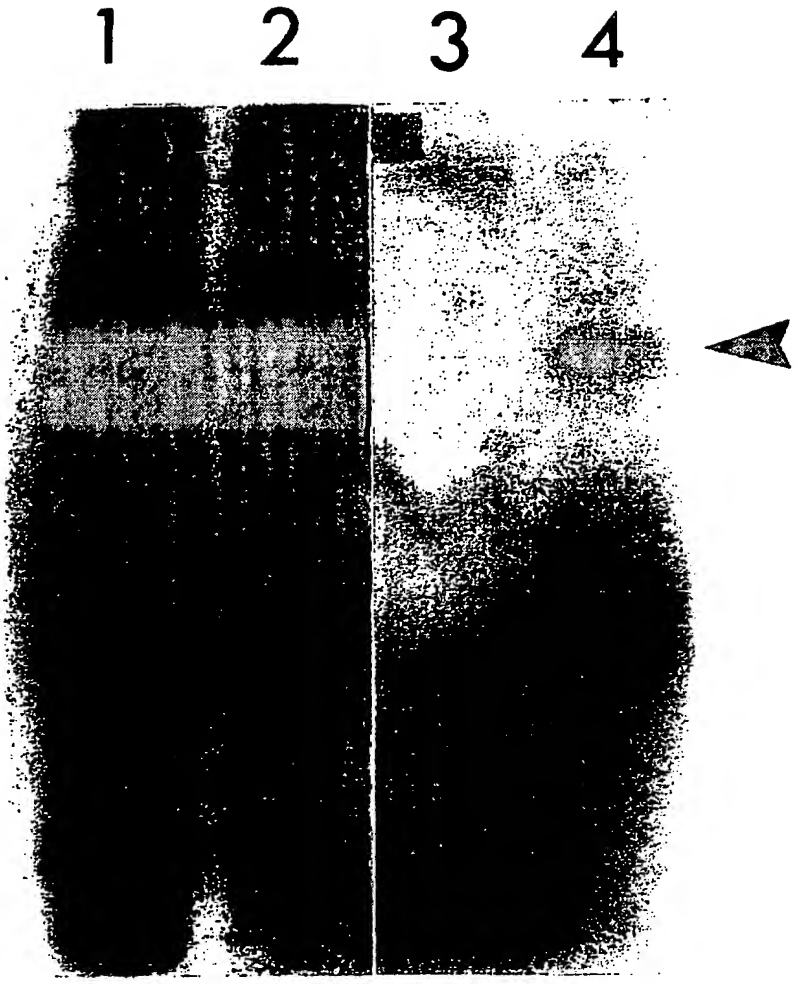

FIG. 1A

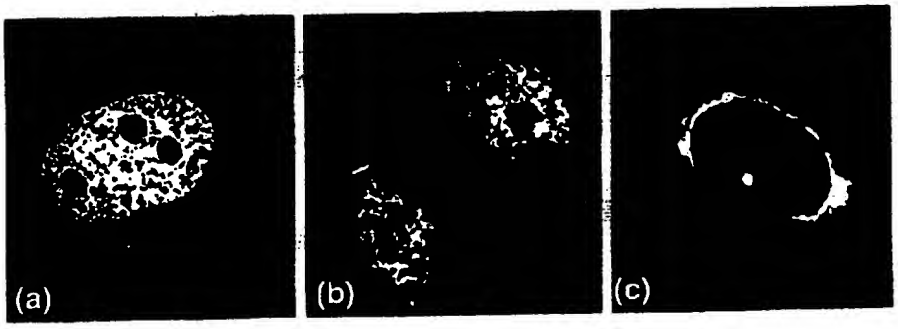

FIG. 1B

AR(µg)	—	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
p60(µg)	—	—	0.11	0.11	0.165	0.165	0.22	0.22	0.33	0.33	0.11	0.165	0.22	0.33	0.33
Peptide	—	—	—	+	—	+	—	+	—	+	—	—	—	—	—
Anti-p60	—	—	—	—	—	—	—	—	—	—	+	+	+	+	NS

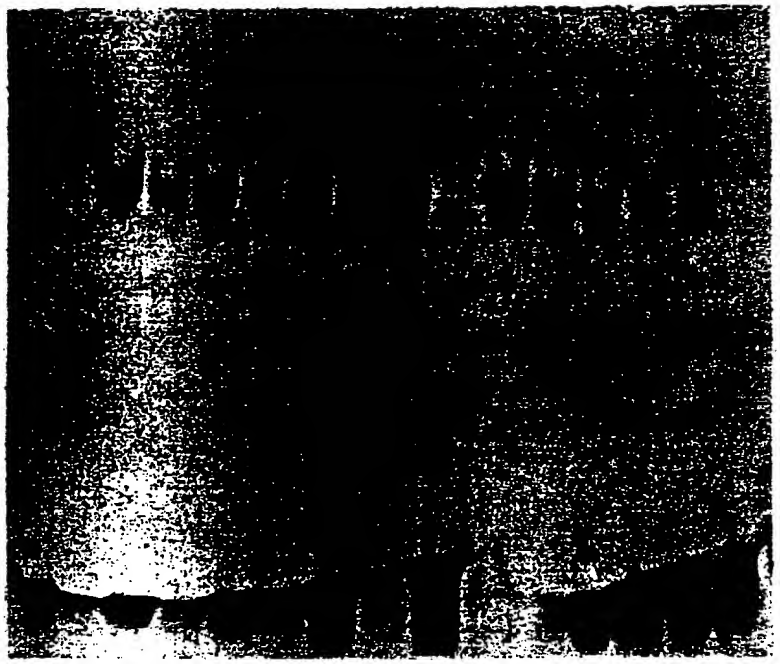

FIG. 2A

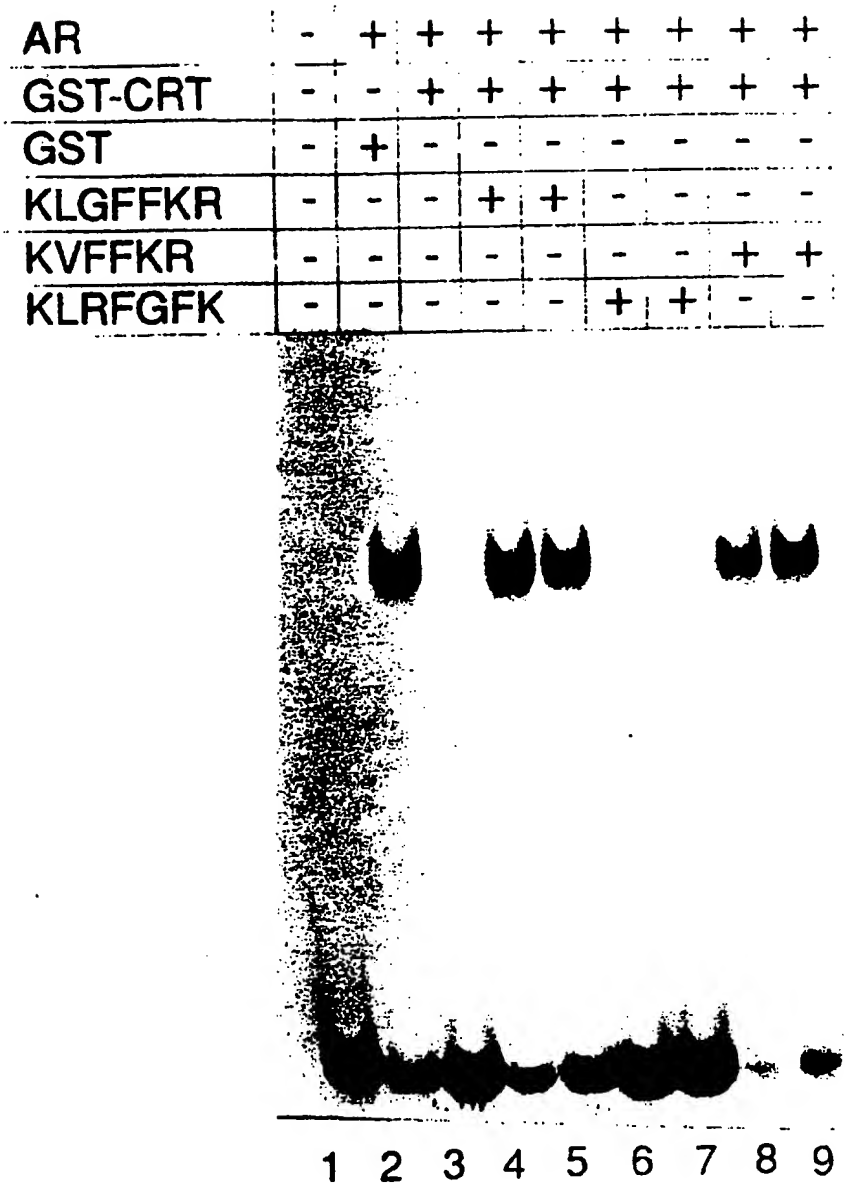

FIG. 2B

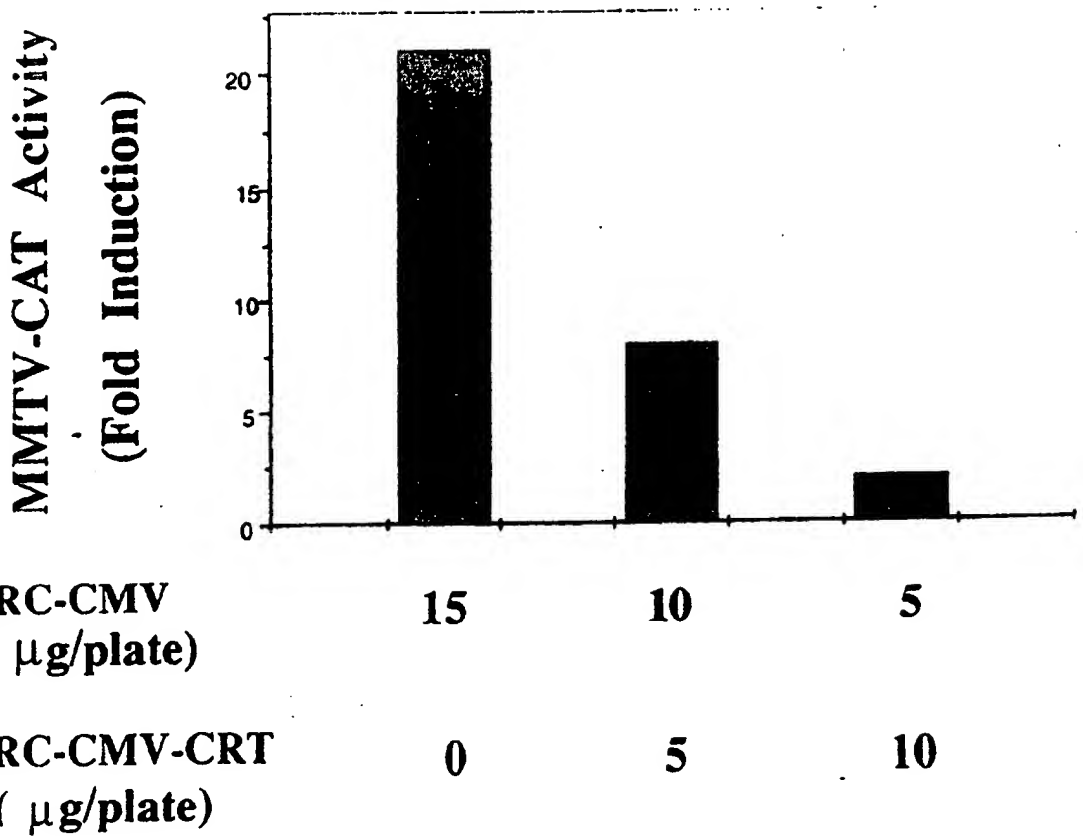
MMTV-CAT Activity
(Fold Induction)
20
15
10
5
0
pRC-CMV
(μg/plate)
15
10
5
pRC-CMV-CRT
(μg/plate)
0
5
10

FIG. 2C

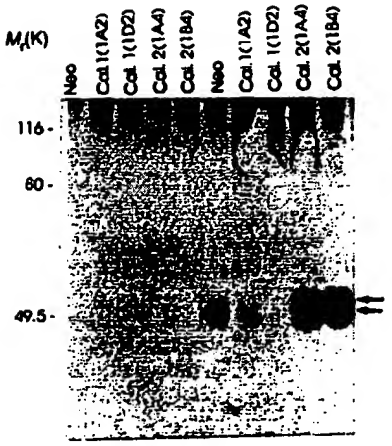
M_r(K)
Neo
Col.1(1A2)
Col. 1(1D2)
Col. 2(1A4)
Col. 2(1B4)
Neo
Col.1(1A2)
Col. 1(1D2)
Col. 2(1A4)
Col. 2(1B4)
116-
80-
49.5-

FIG. 3A

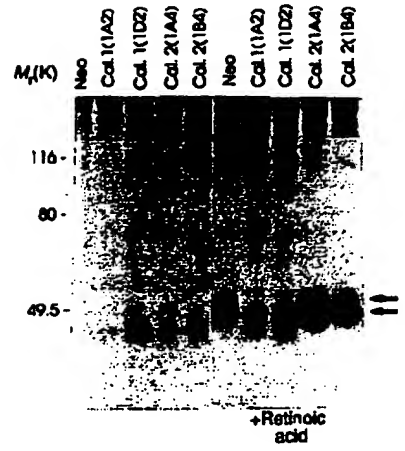
M_r(K)
Neo
Col.1(1A2)
Col. 1(1D2)
Col. 2(1A4)
Col. 2(1B4)
Neo
Col.1(1A2)
Col. 1(1D2)
Col. 2(1A4)
Col. 2(1B4)
116-
80-
49.5-
+Retinoic acid

FIG. 3B

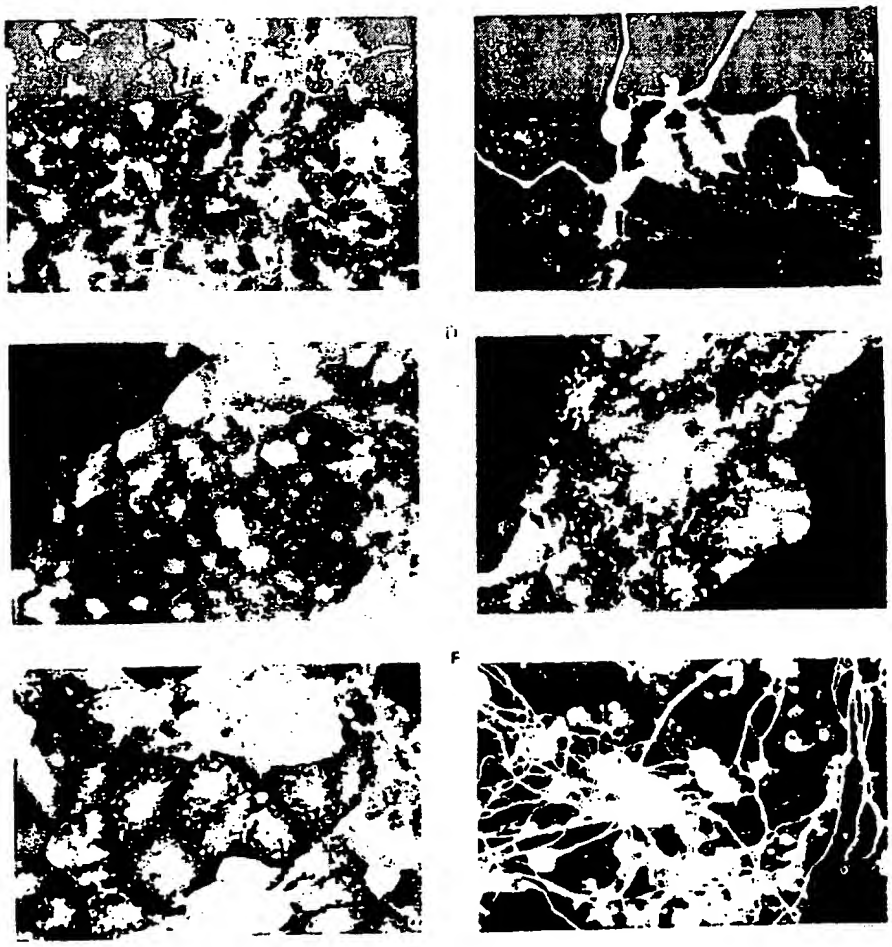

FIG. 3C

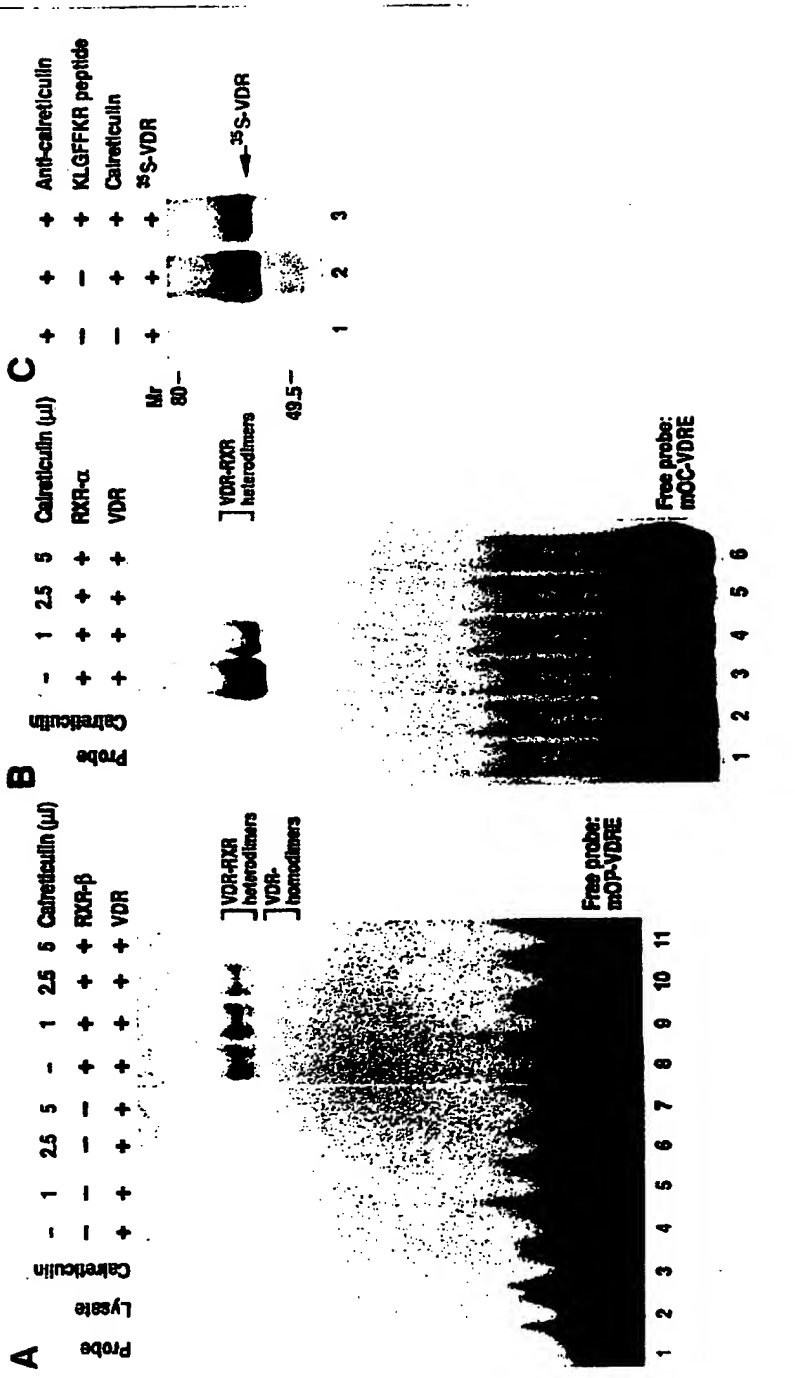
A
Probe
Lysate
Calreticulin
Calreticulin (μl)
RXR-β
VDR
VDR-RXR heterodimers
VDR-homodimers
Free probe: mOP-VDRE
B
Probe
Calreticulin
Calreticulin (μl)
RXR-α
VDR
VDR-RXR heterodimers
Free probe: mOC-VDRE
C
Mr
80
49.5
Anti-calreticulin
KLGFFKR peptide
Calreticulin
35S-VDR
35S-VDR

FIG. 4

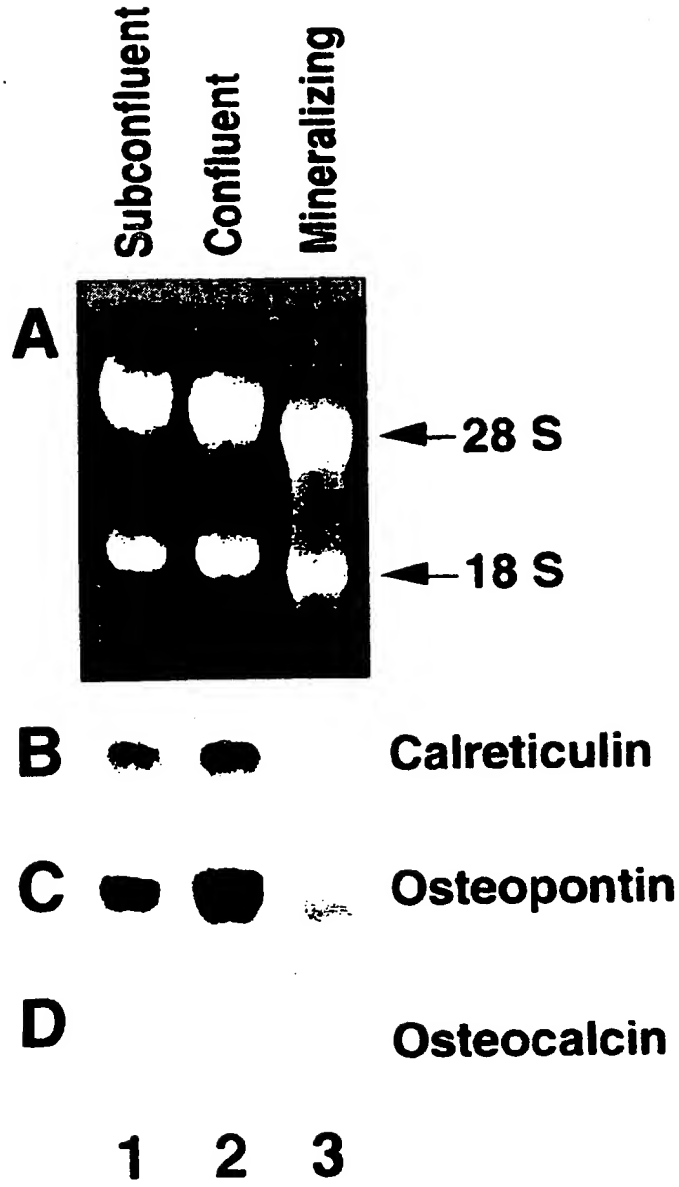
Subconfluent
Confluent
Mineralizing
A
28 S
18 S
B
Calreticulin
C
Osteopontin
D
Osteocalcin
1 2 3

FIG. 5

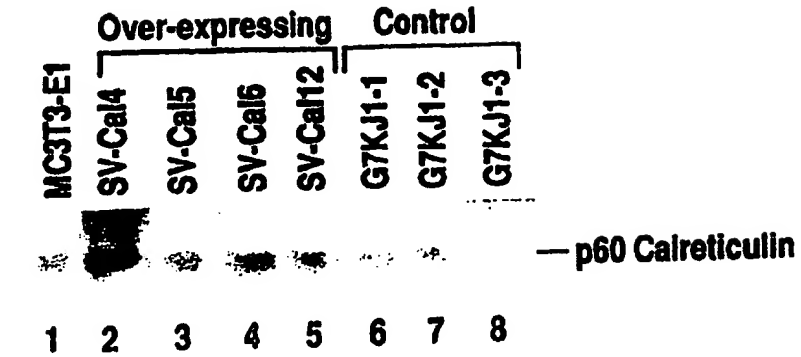
Over-expressing
Control
MC3T3-E1
SV-Cal4
SV-Cal5
SV-Cal6
SV-Cal12
G7KJ1-1
G7KJ1-2
G7KJ1-3
— p60 Calreticulin
1 2 3 4 5 6 7 8

FIG. 6

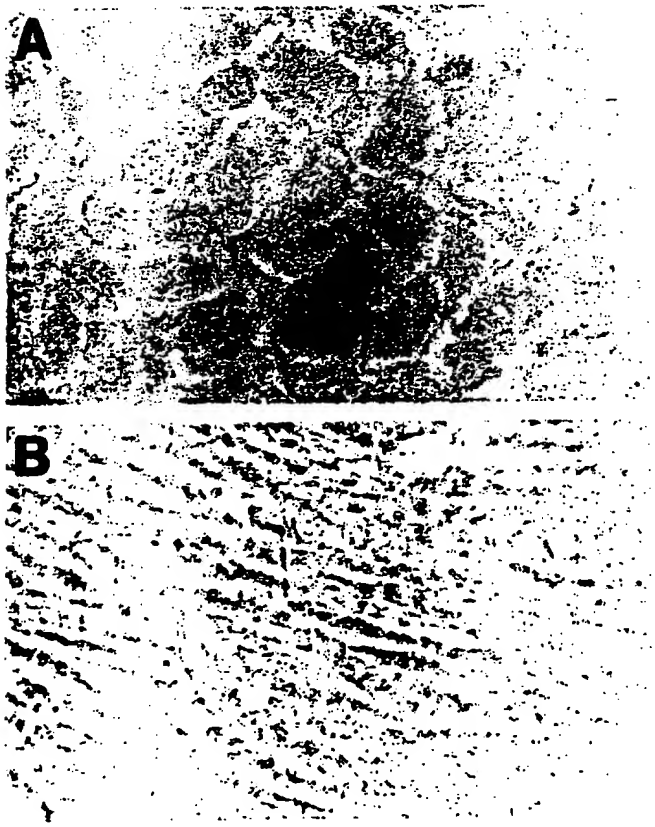
A
B

FIG. 7

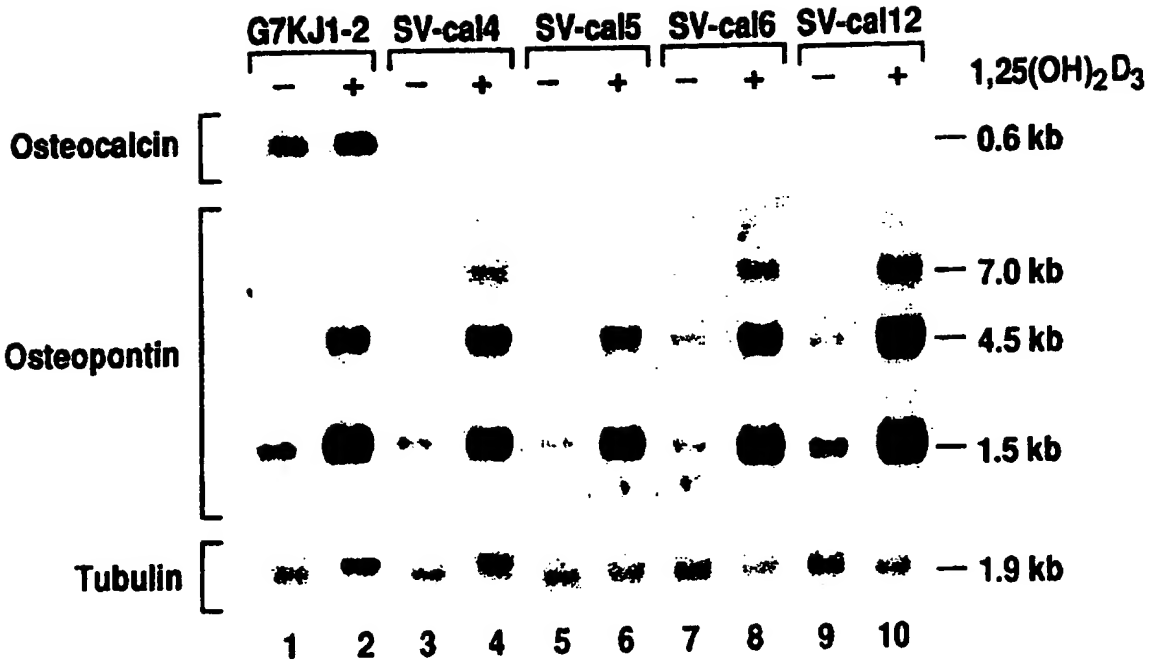
G7KJ1-2
SV-cal4
SV-cal5
SV-cal6
SV-cal12
− + − + − + − + − +
1,25(OH)2D3
Osteocalcin
Osteopontin
Tubulin
— 0.6 kb
— 7.0 kb
— 4.5 kb
— 1.5 kb
— 1.9 kb
1 2 3 4 5 6 7 8 9 10

FIG. 8

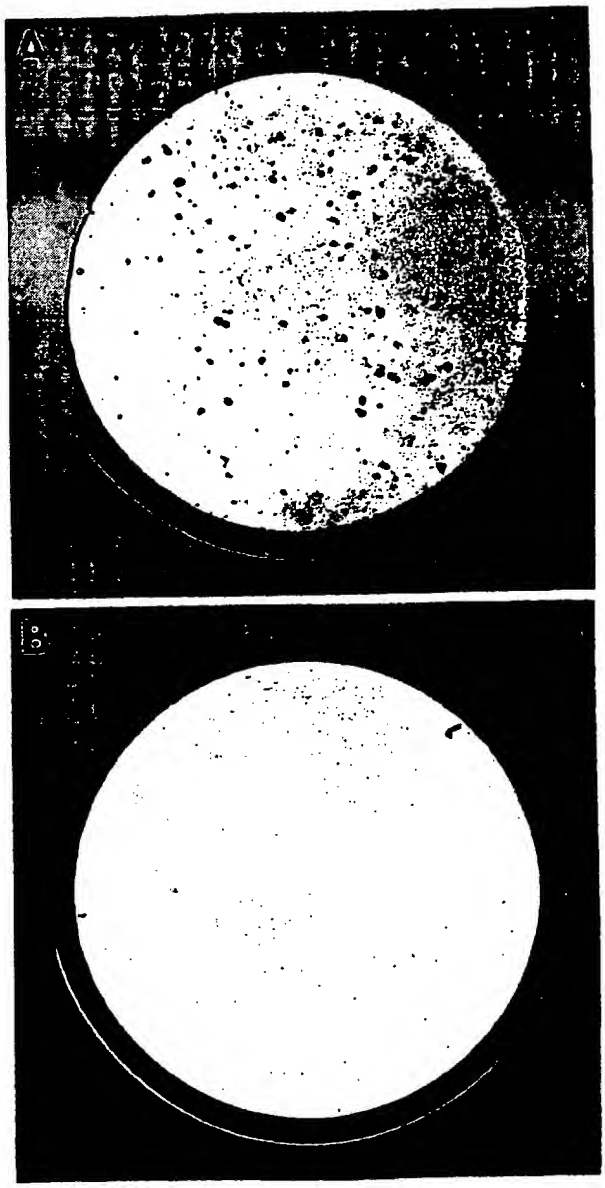

FIG. 9

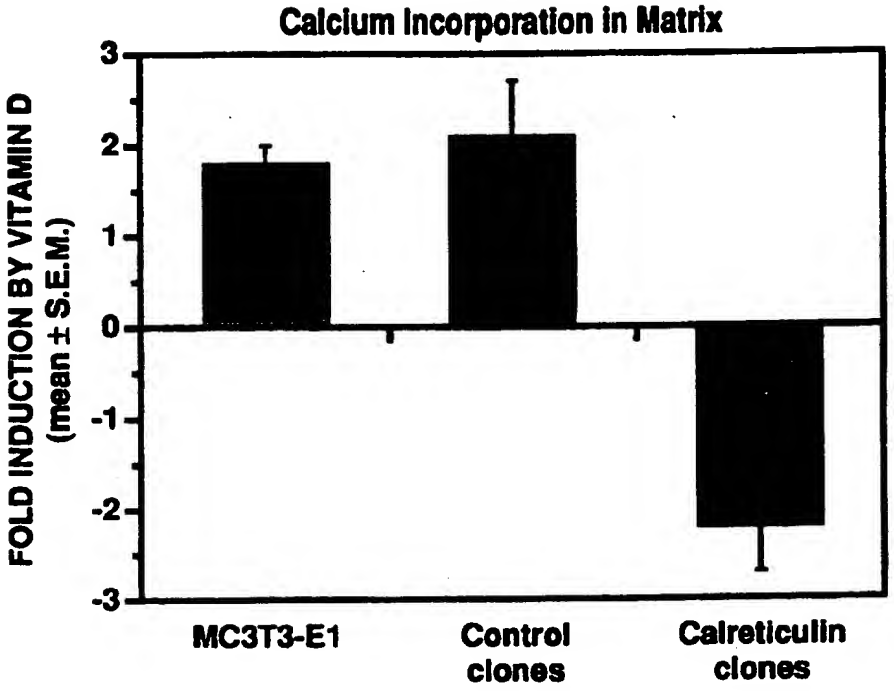
Calcium Incorporation in Matrix
FOLD INDUCTION BY VITAMIN D
(mean ± S.E.M.)
3
2
1
0
-1
-2
-3
MC3T3-E1
Control clones
Calreticulin clones

FIG. 10

INTERNATIONAL SEARCH REPORT

Interna·al Application No
PCT/CA 95/00664

A. CLASSIFICATION OF SUBJECT MATTER

C 07 K 14/575,C 07 K 7/04,A 61 K 38/22,C 12 N 15/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C 07 K,A 61 K,C 12 N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol.367, no. 6462, issued February, 1994, S. DEDHAR et al. "Inhibition of nuclear hormone receptor activity by calreticulin", pages 480-483, the whole article. --	1-11
A	THE BIOCHEMICAL JOURNAL, vol. 285, issued 1992, M. MICHALAK et al. "Calreticulin" pages 681-692, the whole article. ----	

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Date of the actual completion of the international search	Date of mailing of the international search report
26 February 1996	22.03.96

Name and mailing address of the ISA	Authorized officer
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